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Par
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Reverse genetic studies of

Benyvirus – Polymyxa betae molecular interaction:

Role of the RNA4-encoded protein in virus transmission

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PhD Thesis Resume

Introduction

Beet necrotic yellow vein virus (BNYVV), the leading infectious agent that affects sugar beet, is included within viruses transmitted through the soil from plasmodiophorid as *Polymyxa betae*. BNYVV is the causal agent of Rhizomania, which induces abnormal rootlet proliferation and is widespread in the sugar beet growing areas in Europe, Asia and America; for review see (Peltier et al., 2008). In this latter continent, *Beet soil-borne mosaic virus* (BSBMV) has been identified (Lee et al., 2001) and belongs to the benyvirus genus together with BNYVV, both vectored by *P. betae*. BSBMV is widely distributed only in the United States and it has not been reported yet in others countries. It was first identified in Texas as a sugar beet virus morphologically similar but serologically distinct to BNYVV. Subsequent sequence analysis of BSBMV RNAs evidenced similar genomic organization to that of BNYVV but sufficient molecular differences to distinct BSBMV and BNYVV in two different species (Rush et al., 2003). Benyviruses field isolates usually consist of four RNA species but some BNYVV isolates contain a fifth RNA. RNAs 1 contains a single long ORF encoding polypeptide that shares amino acid homology with known viral RNA-dependent RNA polymerases (RdRp) and helicases. RNAs 2 contains six ORFs: capsid protein (CP), one readthrough protein, triple gene block proteins (TGB) that are required for cell-to-cell virus movement and the sixth 14 kDa ORF is a post-translation gene silencing suppressor. RNAs 3 is involve on disease symptoms and is essential for virus systemic movement. BSBMV RNA3 can be trans-replicated, trans-encapsidated by the BNYVV helper strain (RNA1 and 2) (Ratti et al., 2009). BNYVV RNA4 encoded one 31 kDa protein and is essential for vector interactions and virus transmission by *P. betae* (Rahim et al., 2007). BNYVV RNA5 encoded 26 kDa protein that improve virus infections and accumulation in the hosts.

We are interest on BSBMV effect on Rhizomania studies using powerful tools as full-length infectious cDNA clones. B-type full-length infectious cDNA clones are available (Quillet et al., 1989) as well as A/P-type RNA 3, 4 and 5 from BNYVV (unpublished). A-type BNYVV full-length clones are also available, but RNA-1 cDNA clone still need to be modified. During the PhD program, we start production of BSBMV full-length cDNA clones and we investigate molecular interactions between plant and Benyviruses exploiting biological, epidemiological and molecular similarities/divergences between BSBMV and BNYVV.

Material and method

Sugar beet plants were grown on BSBMV infected soil. Total RNA was extracted from infected sugar beet root using Trizol reagent. Virus RNA was synthesized by ImProm-II Reverse Transcriptase system and molecular cloning on bacteria has been used to obtained full length infectious cDNA clones. Using different viral replicons based on BNYVV RNAs (Rep3 or Rep5) we cloned nucleotide sequences of Benyviruses RNA4's ORFs combined with Flag or HA tags in order to investigate expression on different plants (*Chenopodium quinoa*, *Tetragonia expansa* and *Spinacia oleracea*)

During PhD we developed new transmission test on *Beta vulgaris* plants using avirulifer *P. betae* in order to investigate the essential role of BSBMV RNA-4 and p32 protein expression for BNYVV RNA1, 2 and 3 transmissions by *P. betae*.

During molecular characterization of BSBMV p32 protein, we used agroinfiltration system on *Nicotiana benthamiana* to investigate protein subcellular localization fuse with GFP tag. We expressed BSBMV p32 fused with Halo tag on *E. coli* for it purification and antiserum production.

Results and Discussion

We obtained full length infectious cDNA clone of BSBMV RNA-1 and we demonstrate that they transcript is able to substitute BNYVV RNA-1 as it is replicated and packaged *in planta* in a chimeric viral progeny with BNYVV RNA-2. During full-length infectious cDNA clone of BSBMV RNA-2 we identified, by 5'-RACE PCR from BSBMV total RNA extraction, the correct nucleotide sequence at 5' non-coding region. We identified one amino-acid substitution in BSBMV RNA-2 encoded p13 movement protein ($M^{31}V$) that maybe prevents virus cell to cell movement in plant. We reverted this mutation without obtaining full-length infectious clone. Recently we investigated if p15 movement protein is correct expressed and we demonstrated that BSBMV p15 encoded by RNA2 could be complemented by BNYVV p15 or BSBMV p15 expressed by Rep5 viral replicon. Sequence analysis revealed one nucleotide deleted in our full-length cDNA clone of BSBMV RNA-2, PCR site mutagenesis of this nucleotide permitted to obtain a full-length infectious cDNA clone. We demonstrated that transcripts of BSBMV RNA-2 is able to substitute BNYVV RNA-2 as it is replicated and packaged *in planta* in a chimeric viral progeny with BNYVV RNA-1.

During BSBMV full-length cDNA clones production, unexpected 1,730 nts long form

of BSBMV RNA-4 has been detected from sugar beet roots grown on BSBMV infected soil. Sequence analysis of the new BSBMV RNA-4 form revealed high identity (~100%) with published version of BSBMV RNA-4 sequence (NC_003508) between nucleotides 1-608 and 1,138-1,730, however the new form shows 529 additionally nucleotides between positions 608-1,138 (FJ424610). Two putative ORFs has been identified, the first one (nucleotides 383 to 1,234), encode a protein with predicted mass of 32 kDa (p32) and the second one (nucleotides 885 to 1,244) express an expected product of 13 kDa. As for BSBMV RNA-3 (Ratti et al., 2009), full-length BSBMV RNA-4 cDNA clone permitted to obtain infectious transcripts that BNYVV viral machinery (Stras12) is able to replicate and to encapsidate *in planta*. Moreover, we demonstrated that BSBMV RNA-4 can substitute BNYVV RNA-4 for an efficient transmission through the vector *P. betae* in *Beta vulgaris* plants, demonstrating a very high correlation between BNYVV and BSBMV. At the same time, using BNYVV helper strain, we studied BSBMV RNA-4's protein expression *in planta*. We associated a local necrotic lesions phenotype to the p32 protein expression onto mechanically inoculated *C. quinoa*. Flag or GFP-tagged sequences of p32 and p13 have been expressed in viral context, using Rep3 replicons, based on BNYVV RNA-3. Western blot analyses of local lesions contents, using FLAG-specific antibody, revealed a high molecular weight protein, which suggest either a strong interaction of BSBMV RNA4's protein with host protein(s) or post translational modifications. GFP-fusion sequences permitted the subcellular localization of BSBMV RNA4's proteins. Moreover we demonstrated the absence of self-activation domains on p32 by yeast two hybrid system approaches. We also confirmed that p32 protein is essential for virus transmission by *P. betae* using BNYVV helper strain and BNYVV RNA-3 and we investigated its role by the use of different deleted forms of p32 protein. Serial mechanical inoculations of wild-type BSBMV on *C. quinoa* plants were performed every 7 days. Deleted form of BSBMV RNA-4 (1,298 bp, GenBank ID: JF693322) appeared after 14 passages and its sequence analysis show deletion of 433 nucleotides between positions 611 and 1044 of RNA4 new form. We demonstrated that this deleted form can't support transmission by *P. betae* using BNYVV helper strain and BNYVV RNA-3, moreover we confirmed our hypothesis that BSBMV RNA-4 described by Lee et al. (2001) is a deleted form. Interesting after 21 passages we identify one chimeric form of BSBMV RNA-4 and BSBMV RNA-3 (1,142 bp, GenBank ID: JF513084). Two putative ORFs has been identified, the first one (nucleotides 383 to 562), encode a protein with predicted mass of 7 kDa (p7), corresponding to the N-terminal of p32 protein encoded by BSBMV RNA-4; the second one (nucleotides 562 to 789) express an expected product of 9 kDa (p9) corresponding to the 76 C-terminal amino acids of the second putative ORF of BSBMV RNA-3 described by Ratti et

al. (2009). We demonstrated that this chimeric RNA is replicated by BNYVV viral machinery in the presence of BNYVV RNA-3, this results open the prospect to obtain a new viral vector able to to express different endogenous protein and escape from Rep5, Rep3 or RepIII (Ratti et al., 2009) competition for replication.

Conclusion and prospect

We demonstrated that transcript of full length infectious cDNA clone of BSBMV RNA1 can complement BNYVV RNA-1 for an efficient replication of BNYVV RNA-2 and that they can produce chimeric viral progeny *in planta*. These confirm a strong correlation between BNYVV and BSBMV. Moreover we demonstrated that just BSBMV p32 or BNYVV p31 protein expression can support BNYVV RNA-1, 2 and 3 transmission through vector *P. betae* on *Beta vulgaris* plants. We start molecular characterization of BSBMV p32 and our analysis revealed a complex expression profile, with glycosylation, myristoylation and maybe host protein(s) interaction that need to be better investigate in the future.

A paper concerning essential role of BSBMV RNA-4 p32 protein expression on vector transmission of the virus will be soon submitted for publication. Results obtained by our research in this topic, opened new research lines that our laboratories will develop in a closely future. In particular BSBMV p32 and its mutated forms will be used to identify factors, as host or vector protein(s), involved in the virus transmission through *P. betae*. The new results could allow selection or production of sugar beet plants able to prevent virus transmission then able to reduce viral inoculum in the soil.

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GENERAL INTRODUCTION

Sugar beet

Two plants are basically grown in the world for sugar production: sugarcane (*Saccharum officinarum*) in tropical and subtropical climates and sugar beet (*Beta vulgaris*) in temperate climates.

Sugar beet, one of the main industrial crop species, occupies globally a cultivated area of about 8.1 million hectares spread over 41 countries (Rush et al., 2006). The history of crop domestication has been characterized by a short, fast and interesting evolution with pronounced improvements in breeding and cultivation. The potential root sugar content has reached 15-20% throughout the years, today provides approximately 25% of world sugar consumption (FAO, 2009).

In the last years, an additional process of sugar beet cultivation found a great expansion in ethanol production. The recent interest for bioethanol production, as a replacement of fossil fuels in the transportation sector, has triggered significant research efforts in exploiting the crop's potential for production of biofuel and biogas as well.

The cultivated sugar beet is biennial: it develops the vegetative phase during the first year as a near-rosette plant and develops a large fleshy tap root that contains the nutrients reserve for the second year of growth. In the second year the plant begin the reproductive phase, after flowering induction by the combined exposure to low temperatures during winter and long photoperiods during the second year of growth (Milford, 2006).

1.1 Brief history of Sugar Beet cultivation

Sugar beet belongs to the genus *Beta* of the *Chenopodiaceae* family morphologically characterized by inconspicuous, radially symmetric, petal less flowers and non-fleshy fruit (Francis, 2006). All cultivated beet species derive from sea beet (*Beta vulgaris* L. ssp. *maritima*). The genus *Beta* seems to be originated in a widespread area ranging from the British Isles and the North Atlantic coast across from the Mediterranean and the Black Sea as far as the Persian Gulf and the mouth of the Indus River (Biancardi, 2005).

Sea beet domestication is believed to have begun in prehistoric times, around the Persian Gulf as early as wheat and barley, about 12,000 years ago (Simmonds, 1976). Aristophanes (445-385 BC) and Euripides (480-406 BC) provided the earliest references of the beet

plant. From Greece the plant spread in Italy where it was referred to by the Latin name beta, later used by Linnaeus to identify the taxonomic genus (Biancardi, 2005).

With the expansion of the Romans Empire, during the Middle Ages, beet cultivation was highly valued for its edible leaves and sweet-tasting root in particular from Northern Europe people. The history of sugar beet probably starts when the French botanist Olivier de Serres on 1590 extracted sweet syrup from beet roots (Dureau, 1886), however it was only during the second half of the eighteenth century, with Andreas Siegmund Marggraf, a Prussian chemist, that its potential as industrial sugar crop was discovered. He demonstrated that sugar crystals extracted from beet roots were exactly the same as those from sugar cane and his successor, Franz Carl Achard, increase sugar content of beets following several cycles of mass selection leading develop of "White Silesian", the ancestral variety of all modern sugar beet cultivars, whose sugar content ranged from 5 to 7% of total fresh root weight(Coons, 1936). For his results, Achard was supported, by king Frederick William III, to build the world's first sugar beet factory at Cunern in Silesia on 1801 (Winner, 1993).

On the history of modern sugar industry Napoleon played a key role. On 1806, the blockade of sugar cane British imports to France, led Napoleon to find a different way for the supply of sugar in France and he adopted a policy that encouraged the large-scale sugar beet cultivation for sugar production in a growing number of continental European countries. Within the end of the nineteenth century about 400 sugar factories were operating in France, Germany, Austria-Hungary, Bohemia, Czechoslovakia and Russia(Biancardi, 2005). In the middle of the twentieth century, crop productivity was significantly improved: Philippe Andre de Vilmorin introduced the progeny testing into breeding practice and achieved further sugar yield increase of "White Silesian" by means of mass (Coons, 1936).

In the American continent the first sugar beet factory was built in the United States in 1838, in Northampton (Massachusetts), but was closed after its first campaign. After several failed attempts in various states, the first regularly operating factory was built in Alvarado, California. In the new century, sugar beet cultivation gradually spread to other countries: Italy, England, Ireland, Canada, Chile, Uruguay, Japan, Turkey, China etc. In the recent years, it has been introduced into warmer climates that are more suitable for sugar cane cultivation, such as Egypt, Morocco, Tunisia and Algeria (Biancardi, 2005).

Since 2009, within the European Union, the nearly two millions of hectares of sugar beet are mainly produced in Germany and France. Russian Federation, Ukraine and USA also cultivate nearly half million of hectares each year but production of roots per hectare is

quite variable. France, Spain and Belgium, in fact, obtain the higher yield (93.7, 83.57 and 82.69 t/ha, respectively) and Ukraine and Russian Federation the lower (31.49 and 32,32 t/ha, respectively) as reported in Table 1(FAO, 2009).

Country	Area under cultivation (ha)	Root production (t)	Yield (t/ha)
European Union	1,610,520	113,850,731	70.67
Germany	383,600	25,919,041	67.56
France	372,600	34,913,000	93.70
Ukraine	319,700	10,067,500	31.49
Poland	199,936	10,849,200	54.26
United Kingdom	140,000	8,330,000	59.5
Netherlands	72,700	5,735,000	78.88
Belgium	62,700	5,185,100	82.69
Serbia	61,399	2,797,596	45.56
Italy	60,600	3,307,700	54.58
Spain	49,700	4,153,900	83.57
Czech Republic	52,465	3,038,220	57.91
Austria	43,860	3,083,135	70.29
Sweden	39,800	2,405,800	60.44
Russian Federation	770,200	24,892,000	32.32
United State of America	464,827	26,779,190	57.61
Turkey	323,970	17,274,674	53.32
China	220,000	9,500,000	43.18
Iran	54,404	2,041,278	37.52
Japan	64,500	3,649,000	56.57
Egypt	115,000	5,133,513	44.64
World	4,323,671	229,490,296	53.07

Table 1. World sugar beet production. Cultivation area, roots production and yield of the most sugar beet productive country of the World.

1.2 Diseases of sugar beet

A wide range of pathogenic agents (viruses, fungi, bacteria, nematodes and insects) commonly attack sugar beet culture, making in many cases its exploitation unprofitable due to the reduction of both taproot size and sugar content (Whitney and Duffus, 1986). All parts of the plant and all cultivation stages can be targeted by different pathogens.

Consequently, diseases caused by pests of major importance have played a significant role in the current distribution of the crop and of the related sugar industry ((Cesarini, 1999); (Scholten and Lange, 2000)).

From viruses, rhizomania is the most dangerous and worldwide spread disease. This thesis focused on rhizomania disease and on rhizomania related viruses, in particular we compared molecular proprieties and protein functions of two *Benyvirus*: *Beet necrotic yellow vein virus* (BNYVV) and *Beet soil-borne mosaic virus* (BSBMV).

Disease	Causal agent
Viruses	
Rhizomania	*Beet necrotic yellow vein virus* (BNYVV)
Rhizomania related virus	*Beet soil-borne mosaic virus* (BSBMV)
	Beet soil-borne virus (BSBV)
	Beet virus Q (BVQ)
	Beet oak leaf virus (BOLV)
Beet mosaic	*Beet mosaic virus* (BtMV)
Beet yellows	*Beet yellows* (BYV)
	Beet mild yellows (BMYV)
	Beet western yellows (BWYV)
	Beet chlorosis virus (BChV)
Curly top	*Beet curly top virus* (BCTV)
Bacteria	
Bacterial vascular necrosis and rot	*Erwinia carotovora* spp. *betavasculorum*
Bacterial leaf spot or leaf blight	*Pseudomonas syringae*
Yellow wilt	Rickettsia-like organism
Fungi	
Cercospora leaf spot	*Cercospora beticola*
Alternaria leaf blight	*Alternaria alternata, Alternaria brassicae*
Powdery mildew	*Erysiphe betae*
Downy mildew	*Peronospora schachtii* (*farinosa*)
Fusarium yellows / Fusarium root rot	*Fusarium oxysporum* f. sp. *Betae*
	Rhizoctonia solani
Root rots	*Pythium* spp.
	Phoma betae
Southern sclerotium root rot	*Sclerotium rolfsii*
Black root / Black leg	*Aphanomyces cochlioides*
Nematodes	
Cyst nematode	*Heterodera schachtii*
Root-knot nematode	*Meloidogyne* spp.

Table 2. Principal sugar beet diseases.

Rhizomania disease

The word 'rhizomània' is composed by the Latin parts 'rhizo' and 'man˘ıa', derived from the Greek 'ρίζα' and 'μανία' meaning, respectively, 'root (radical)' and 'abnormal trend (madness) (Biancardi, 2005). Rhizomania, meaning "crazy root" or "root madness" was initially describe by Canova (1959), during observations of sugar beet fields in Padan Plain, the border region of Po River, in the mid 50's and has since been reported in all sugar beet producing countries worldwide ((Tamada, 1999);(Lennerfors et al., 2005); (McGrann et al., 2009)).

Symptoms of the viral rhizomania disease are mainly localized on the lower part of the plant. Roots of infected beets appear "bearded" because of an excessive proliferation of lateral rootlets, which causes more or less taproot stunting (Fig. 1). Browning of the vascular system as well as constriction of the main root are visible on beets cross-sections (Fig. 1) ((Brunt and Richards, 1989); (Putz et al., 1990)). The size of the taproot can be strongly reduced, constricted and resembling the shape of a wine glass. Infected roots are inefficient in water and nutrient uptake and therefore the leaves commonly become pale green with long petioles and up right growth. BNYVV is rarely systemically spread to the leaves. When systemic spread occurs, it is mostly manifested by the necrosis and yellowing of the leave veins, that provided the name for the disease causal agent (Tamada, 1975). Disease responses at the physiology level include a reduced transpiration and CO_2 uptake, reduced content of nitrogen, chlorophyll and carotenoid and elevated content of amino nitrogen, sodium and potassium in the root sap ((Keller et al., 1989); (Královic and Králová, 1996); (Steddom et al., 2003)).

Fig. 1. Rhizomania symptoms caused by BNYVV infection on sugar beet. (a) yellowing and necrosis on the leaves veins; (b) lateral rootlets proliferation with browning of the vascular system and constriction of the main root; (c) symptoms on fields.

The disease causes severe economic losses as a consequence of a dramatic reduction in root yield, sugar content and purity, especially when infections occur early in the growing season.Sugar yield reduction commonly around 50-60% and sometimes up to 80% (McGrann et al., 2009).

2.1 The pathogen, Beet necrotic yellow vein virus

Rhizomania disease is caused by the *Beet necrotic yellow vein virus* (BNYVV) (Tamada and Baba, 1973), the type member of *Benyvirus,* a genus remaining un assigned in terms of family classification (Rush, 2003). Benyviruses are all vectored by the plasmodiophorid protozoa *Polymyxa betae* Keskin (Keskin, 1964) and are characterized by multipartite non-enveloped rod-shaped particles with a positive single strand RNA (ssRNA) harbouring a 5' Cap and 3' polyA.

BNYVV RNA-1 and -2 are essential and sufficient for viral multiplication when mechanically inoculated to the leaves of the *Chenopodiacea* plants like *Beta macrocarpa*, *Beta vulgaris*, *Chenopodium quinoa* and *Tetragonia expansa*((Koenig et al., 1986); (Pelsy and Merdinoglu, 1996); (Tamada et al., 1989). These two RNAs carry "house-keeping" genes involved in RNA replication, assembly, cell-to-cell movement and suppression of post-transcriptional gene silencing (PTGS) ((Tamada, 1999); (Dunoyer et al., 2002). This characteristic allowed Quillet et al. (1989) to develop a replicative strain of BNYVV named "Stras12", by extracting total RNA from leaves infected with transcripts from full-length cDNA clones of B type BNYVV RNAs-1 and -2. However, the natural infection process requires the host-specific function of additional proteins encoded by RNAs-3 and -4, directly involved in pathogenesis and vector transmission for the efficient production of typical rhizomania symptoms and virus propagation between plants ((Lemaire et al., 1988); (Koenig et al., 1991)). Field isolates consist of 4 or 5 genomic RNAs whereas laboratory isolates maintained in the greenhouse by repeated mechanical inoculations, often develop internal deletions within RNAs -3 and -4 or may entirely loose these small genome segments(Bouzoubaa et al., 1985);(1991)).

RNA-1 (6,746 nucleotides, nts) contains a single large open reading frame (ORF) that encodes a 237 kDa polypeptide containing the conserved motifs of methyltransferase (MTR), helicase (HEL) and RNA-dependent RNA polymerase (RdRp, POL), the viral RNA replicase which is essential for virus replication (Bouzoubaa et al., 1987). Following translation, p237 is processed, by an autocatalytic cleavage that gives rise to p150 and p66

proteins (Hehn et al., 1997). The first one contains the MTR and HEL motifs and RdRp motif is present in the p66 protein. This proteolytic cleavage of the replicase distinguishes the *Benyviruses* from all other viruses with rod-shaped particles, which have their replication-associated proteins encoded on two ORFs.

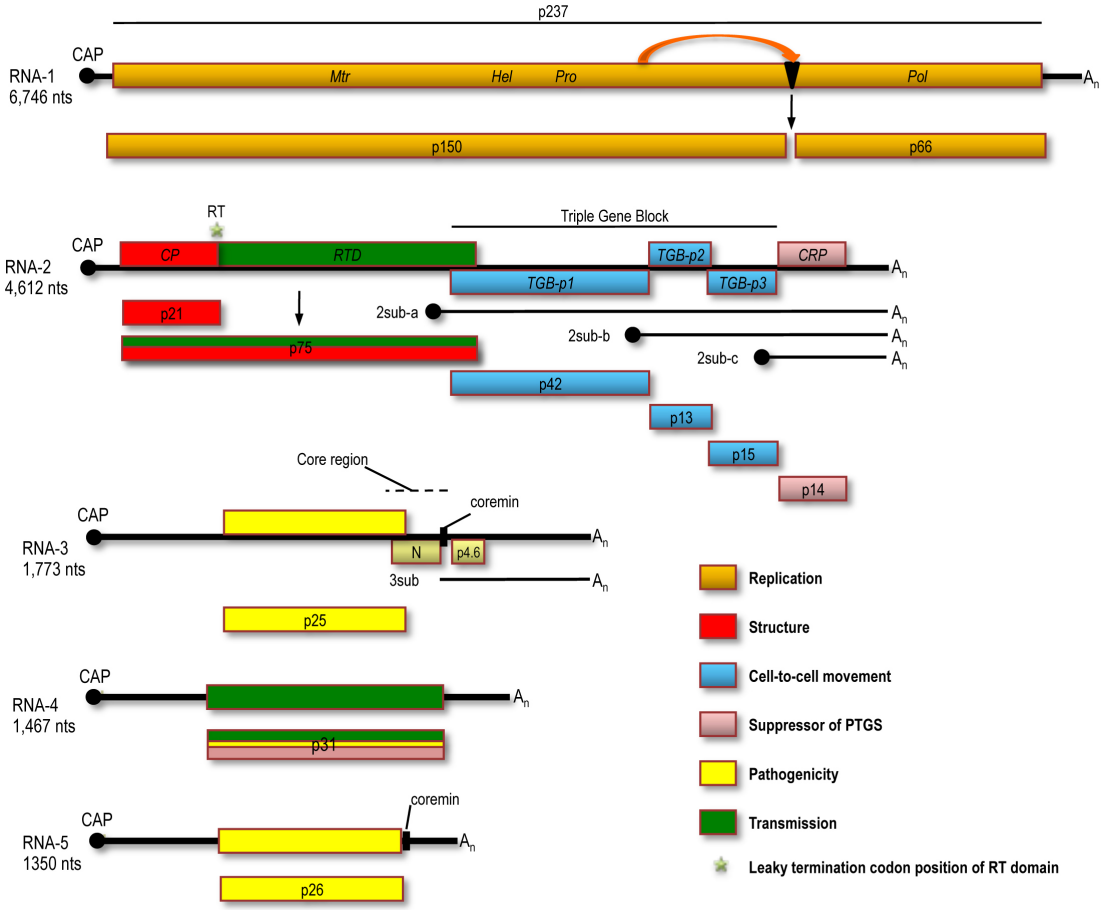


Fig. 2. Schematic representation of the BNYVV multipartite genome composed of four to five single-stranded positive-sense RNAs. All RNAs are 5'-capped and 3'-polyadenylated. Viral replicase contains conserved methyltransferase (MTR), helicase (HEL) and RNA-dependent-RNA polymerase (POL) motifs. The coat protein (CP) and a read-through (RT) domain are encoded by RNA-2 as well as the Triple Gene Block (TGB) proteins. Subgenomic RNAs are formed on RNA-2 and RNA-3. The molecular mass is indicated for each protein in kilodalton (kDa) inside the corresponding ORF. Function(s) attributed to each proteins are specified through a colour code.

RNA-2 (4,609 nts) contains six ORFs. The first ORF encodes the 21 kDa viral Coat Protein (CP), followed by an in-frame region of 54 kDa read-through (RT) domain. CP is fused to RT during translation when the leaky UAG stop codon is bypassed by ribosomes that produce then a 75 kDa CP-RT ((Ziegler-Graff et al., 1985); (Niesbach-Klosgen et al., 1990)). The N-terminal part of the RT domain is involved in viral assembly (Schmitt et al., 1992) whereas the C-teminal part is essential for the viral transmission by *P. betae*(Tamada and Kusume, 1991). The next three partially overlapped ORFs, expressed by two subgenomic

RNAs, encode three protein products (p42, p13, p15) that exhibit typical motifs of the "triple gene block" (TGB) movement proteins, also found among other genera such as *Potexvirus*, *Carlavirus*, *Pomovirus*, *Hordeivirus*, *Allexivirus*, *Foveavirus* and *Peduvirus* (Morozov and Solovyev, 2003);(Verchot-Lubicz et al., 2010), thus facilitating virus cell-to-cell movement (Gilmer et al., 1992). The last ORF is also expressed from a sub-genomic RNA (Gilmer et al., 1992) that encodes a cysteine-rich protein (p14), which possesses a suppressor activity of post-transcriptional gene silencing (PTGS) (Dunoyer et al., 2002).

RNA-3 (1,774 nts) encodes for two pathogenicity-associated proteins of 25 kDa (p25) and 6.8 kDa (N) and, putatively, for a third 4.6 kDa protein. Expression of p25, which has been serologically detected in the cytosolic fraction of infected *C. quinoa* leaves (Niesbach-Klosgen et al., 1990), is associated to the appearance of bright yellow local lesions on *C. quinoa* ((Tamada et al., 1989); (Jupin et al., 1992)), to the development of rhizomania symptoms in sugar beet roots ((Tamada et al., 1990); (Koenig et al., 1991)) and its expression induces abnormal root branching in transgenic *Arabidopsis thaliana* (Peltier et al., 2010). Variability on BNYVV RNA 3-encoded p25 protein, especially at amino acid positions 67-70 (Schirmer et al., 2005) has been recently associated with an increased pathogenicity and resistance breaking in resistant cultivars. Schirmer et al. (2005) suggested the tetrad motif $aa_{67\text{-}70}$ as being responsible for variable virulence with the isolates belonging to the p25-I and p25-II groups presenting the higher variability and pathogenicity rate. Moreover, Acosta-Leal et al. (2008) reported an additional variability in aa position 135 and hypothesized a possible correlation of $V_{67}L_{68}E_{135}$ residues with the ability of these isolates to overcome rhizomania resistance conferred by the *Rz1* resistance gene, derived from "Holly" source ((Lewellen et al., 1987); (Lewellen, 1988)). In addition, the aa composition at positions 129 and 179 has been also correlated with variable pathogenicity and resistance breaking incidence ((Chiba et al., 2008); (Koenig et al., 2009).

The functions of the additional two ORFs (N and 4.6) present in RNA-3 are not well documented. Jupin et al. (1992) reported that ORF N (1,052 – 1,231 nts), which overlaps the 3' terminal portion of the p25 ORF, has the potential to encode a protein of 6.8 kDa that is not detectably expressed from full-length RNA-3 but by spontaneous or experimental deletion of the upstream portion of the p25 ORF. When this protein is express, it induces necrotic local lesions on test plants as *Tetragonia expansa* and *C. quinoa.* The other 4.6 kDa protein has never been detected.

Lauber et al. (1998) identified on BNYVV RNA-3 a nucleotide sequence that is essential for long-distance movement (systemicity) of the virus in *B. macrocarpa*, called "Core region" (1033-1257 nts).

RNA-4 (1,467 nts) is important for efficient transmission of the virus by the plasmodiophorid vector (Tamada and Abe, 1989). Furthermore, Rahim et al. (2007) confirmed that p31 protein expression is required for efficient vector transmission but demonstrated that p31 protein is also involved in enhanced symptom expression in host-specific manner. Indeed, p31 is able to enhance the ability of BNYVV to suppress silencing in roots of *Nicotiana benthamiana* plants (Andika et al., 2005); (Rahim et al., 2007).

When present, RNA-5 encodes for another pathogenicity-associated protein of 26 kDa (Kiguchi et al., 1996). Its function relates to the enhancement of virus transportation through the vascular bundles and an increased symptom severity. Due the aggressiveness of isolates containing RNA 5 (Tamada et al., 1996), it has been proposed that p26 probably acts in a synergistic manner with RNA 3-encoded p25 (Link et al., 2005).

2.2 The vector: Polymyxa betae

The endoparasite *P. betae* is an eukaryotic protist belonging to the *Plasmodiophoridae* family, formerly considered as a fungi group but reclassified as protozoa in the Cercozoa group ((Barr and Asher, 1992); (Braselton, 1995)). *P. betae* infects mainly roots of *Chenopodiaceae* but practical experience suggests that it causes limited damage to the sugar beet crop. However, some differences in virulence between *P. betae* isolates have been reported upon the reduction of roots growth on the sugar beet plant ((Gerik and Duffus, 1988); (Blunt et al., 1991); (Kastirr et al., 1994)). More in general, five forms of another *Polymyxa* species, *P. graminis*, are also reported to vector many viruses in both tropical and temperate regions, in particular on cereals ((Legreve et al., 2000); (Legreve et al., 2002); (Kanyuka et al., 2003); (Dieryck et al., 2009)).

Life cycles of both *Polymyxa* species are highly similar and *P. betae* cycle is outlined on Fig. 2. Clusters of thick-walled resting spores, termed cystosori (Fig. 3), are released into the soil during senescence and decline of infected plant roots. In the presence of a susceptible host, warm and near-saturated soil moisture conditions, resting spores germinate and release primary zoospores, a motile spore that encyst on rootles and during infection inject their cytoplasmic content inside root cells inducing the formation of a multinucleate plasmodium. During this stage virus particles can be transferred to the host or acquired by the vector. Plasmodium can be differentiates in a zoosporangium or sporosorus. The sporangial phase leads to immediate production of secondary zoospores leading new infection cycle, whereas the sporogenic phase ensure cystosori release with long-term

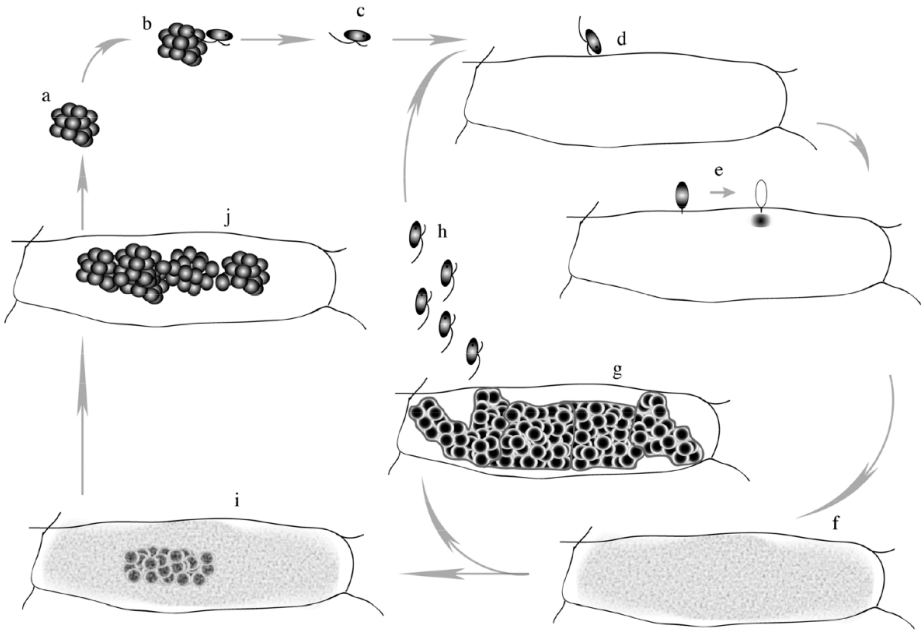
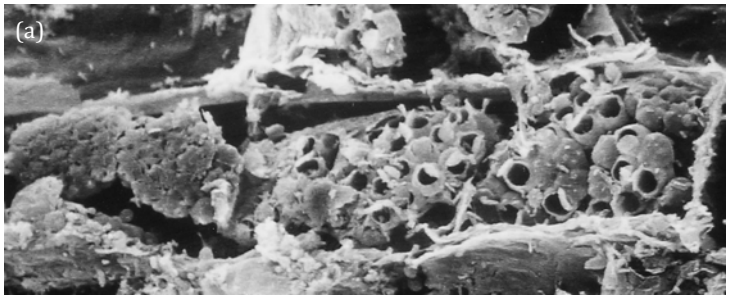


Fig. 2. Schematic representation of the *P.betae* life cycles and its developing states. (a) cystosori also called "spore balls"; good condition induce germinating of zoospore (b); swimming zoospores (c) is positively attracts by cortical or epidermal cells (d); (e) the zoospore encyst on the cell and injects its contents through the cell wall and the cellular membrane inducing plasmodium develop (f) that will ten to a zoosporangium (g) that will issue either (h) the secondary zoospores (h) able to infect new cells or (i) to the sporogenous plasmod (j) leading to new cystosori that will be further released in soil after root decomposition (Adapted from Peltier et al. 2008).



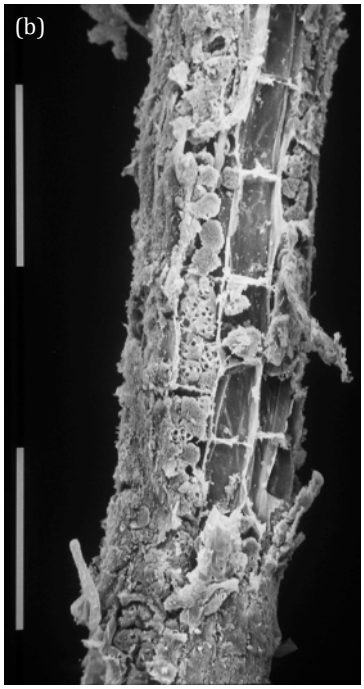
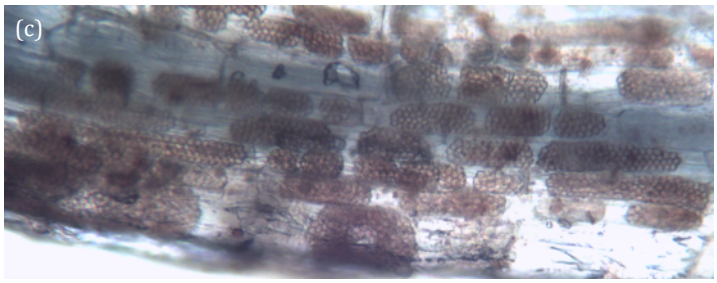


Fig. 3. Scanning Electron Microscope (SEM) ((a) and (b)) and optical microscope (c) images of *P. betae* resting sporesin sugar beet cortical cells roots. (c) Optical microscope images of *P. betae* resting spores.



persistence in the soil (20 – 25 years). Under optimal conditions, soil pH between 6.0 and 8.0, high water content and a temperature of + 25°C, the infection cycle is completed within 60 hours during the sporangial phase. One or the other phase is preferred depending on the external conditions or the host type ((Keskin, 1964); (Asher and Blunt, 1987)). Vectoring the rhizomania-responsible viruses, *P. betae* allows the dissemination of one of the worst viral disease of beet. Recently, Lubicz et al. (2007) have provided proof that BNYVV accumulates in resting spores and zoospores of its vector. Moreover, associations of the viral replication and movement proteins with sporangial and sporogenic stages of the plasmodiophorid vector led these authors to conclude that the virus resides inside its vector for more than a life cycle and to further suggest that *P. betae* besides being a vector may have an additional role as a host.

P. betae is also the vector for other soil-borne sugar beet viruses as BSBMV, BSBV and BVQ that can be transmitted in a single or mixed infection often associated to BNYVV.

2.3 BNYVV variability

The study of BNYVV genome by restriction fragment length polymorphism (RFLP), single-strand conformation polymorphisms (SSCP) or sequence analysis of reverse transcription and polymerase chain reaction (RT-PCR) products from RNA-1 to -5, allowed to identify three major types of the virus named A, B, and P ((Kruse et al., 1994); (Koenig et al., 1995). Afterwards, partial or complete sequencing combined with phylogenetic analysis allowed the classification of BNYVV isolates also according to their pathogenicity, sequence diversity and geographic origin ((Koenig and Lennerfors, 2000); (Meunier et al., 2003);(Schmidlin et al., 2005)).

Types A and B are distributed worldwide and contain only four RNA species. Currently, type A is widespread in most European countries, the USA, China and Japan. Type B has a limited spread and has been found in Germany, France, UK, Belgium, Sweden, China, Japan, Lithuania, The Netherlands and Iran ((Miyanishi et al., 1999);(Lennerfors et al., 2000); (Sohi and Maleki, 2004);(Ratti et al., 2005))

Variations from 3% to 6% have been observed between nucleotide sequences of A and B types but a sequence identity of more than 99% suggests a highly conserved genome among isolates within A and B types ((Koenig and Lennerfors, 2000); (Meunier et al., 2003)).To date, the molecular discrimination between A and B types does not seem to be related to different pathogenicity in contrast with the P type that is known to be

responsible for severe rhizomania symptoms even in resistant varieties ((Heijbroek et al., 1999)). Type P has been identified in France (near Pithiviers) and contains 5 RNAs ((Kruse et al., 1994); (Koenig et al., 1995)) but isolates containing the fifth RNA have been reported earlier from Japan ((Tamada et al., 1989); (Kiguchi et al., 1996)) and later from China (Dawei et al., 1999), Kazakhstan (Koenig and Lennerfors, 2000) and Lennerfors, 2000), UK ((Harju et al., 2002); (Ward et al., 2007)), Germany, where an Asian-like BNYVV isolate containing 5 RNAs has been recently identified (Koenig et al., 2008) and more recently in Iran (Mehrvar et al., 2009).

According to nucleotide sequence analysis the P type isolates result related more closely to the A type than the B type isolates. Moreover sequence comparisons of RNA 5-encoded p26, led to the establishment of three groups (I, II and III) of isolates with a fifth RNA species; most isolates from Japan and China belong to group I, two isolates from Japan constitute group II and the French isolates are included in group III (Miyanishi et al., 1999).

More recently Chiba et al. (2011) attempted to explain the evolutionary history and route of BNYVV spreading. The authors proposed and early divergence of the A and B lineages isolates followed by loss of RNA-5 genome component in all B lineage isolates and some A lineage population. Comparing nucleotide sequence of CP, p25 and p31 protein from 73 BNYVV isolates collected worldwide they identified eight clusters that are best distinguished by the p25 sequence, derived from four original BNYVV populations variant and their mixed infections.

Beet soil-borne mosaic virus (BSBMV)

Beet soil-borne mosaic virus (BSBMV) is a member of *Benyvirus* genus (Lee et al., 2001). It was first identified in Texas as a sugar beet virus morphologically similar but serologically distinct to BNYVV (Liu and Duffus, 1988).

Rush et al. (1994) demonstrated that PCR primers designed for the 3' end of each BNYVV RNA species amplify homologous regions of BSBMV and when these BSBMV PCR products were used as probes in Northern blots (Heidel et al., 1997), they weakly hybridized with BNYVV. In contrast, PCR specific primers designed on the 5' end of BNYVV RNAs do not amplify BSBMV.

When BSBMV was for first time completely sequenced by Lee et al. (2001), it was determined that BSBMV and BNYVV have identical genomic organization but sufficient molecular differences to be distinguished in two different species.

RNA-1 is 6,683 nts long and contains a single long ORF encoding a 239 kDa product that shares amino acid homology with known viral MTR, HEL and RdRp.

RNA-2 is 4,615 nts long and contains six ORFs. The 21 kDa viral capsid protein (CP) ORF is located at the 5' terminus followed by a leaky UAG stop codon, whose suppression leads to the expression of the 74 kDa RT protein. The next three BSBMV RNA-2 ORFs have typical motifs of TGB and the sixth 14 kDa protein is also similar to the cysteine-rich protein of BNYVV, which regulates RNA-2 and CP accumulation ((Hehn et al., 1995); (Lee et al., 2001)).

The 1,720 nts BSBMV RNA-3 encodes the 29 kDa ORF that shares 23% amino acid sequence identity with the 25 kDa ORF of BNYVV RNA-3. The role of BSBMV RNA-3 on symptoms determination on *C. quinoa* plants has been recently described (Ratti et al., 2009).

Lee et al. (2001) described a single putative ORF on the 1,203 nts BSBMV RNA-4 with a predicted mass (13 kDa) considerably smaller than the BNYVV RNA-4 31 kDa product.

Sequence analyses confirmed that specific regions of the BSBMV genome exhibited high degrees of nucleotide homology with BNYVV, whereas other regions were quite different. The predicted ORFs on BNYVV and BSBMV reveal 23% (RNA-3 ORF) to 83% (RNA-1 ORF) amino acid identity and the nucleotide identity score range between 35% and 77%. This results show that BNYVV and BSBMV viruses are distinct, yet more closely related to each other than to any of the other rod-shaped multipartite viruses with "fungal" vectors from the original genus *Furovirus* (Lee et al., 2001).

BSBMV is widely distributed only in the United States and, up to date, it has not been reported in other countries ((Rush, 2003); (Ratti et al., 2009)). Roots of BSBMV-infected sugar beets are in general asymptomatic, but when the virus systemically spreads to the leaves, foliar symptoms may be seen including yellow vein-banding, mottling or slightly disordered growth (Heidel and Rush, 1994). Pre-infection with BSBMV reduced significantly BNYVV titre (Mahmood and Rush, 1999). Moreover studies on separate and mixed infections of BSBMV and BNYVV have revealed that, upon single infection, BSBMV causes a reduction in fresh root weight, whereas in mixed infections with BNYVV its pathogenic effect is less pronounced due to a suppressive action by the latter (Wisler et al., 2003).

Aim of the study

Beet necrotic yellow vein virus (BNYVV) and, *Beet soil-borne mosaic virus* (BSBMV) belong to the *Benyvirus* genus and both vectored by *P. betae*. BSBMV is widely and essentially distributed in the United States and has never been reported in others countries yet. Sequence analysis of BSBMV RNAs evidenced similar genomic organization to that of BNYVV but sufficient molecular differences to distinct BSBMV and BNYVV in two different species not serologically related.

The studies performed during my PhD have been focused on BSBMV and thus I started BSBMV studies using powerful tools as full-length infectious cDNA clones that allowed investigation of molecular interactions between plant and Benyviruses exploiting biological, epidemiological and molecular similarities/divergences between BSBMV and BNYVV, using different viral replicons based on BNYVV and BSBMV RNAs (Rep3, Rep5 and RepIII) and tagged protein expression in different plants (*C. quinoa*, *T. expansa* and *Spinacia oleracea*).

Experiments performed and results obtained during my PhD are described in chapter 1, 2 and 3 of this thesis. In chapter 1 production of BSBMV full-length cDNA clones, sequence comparison and function complementation between other *Benyvirus* are reported and then discussed. Chapter 2 is presented as a paper project where identification, role on long distance movement, symptoms expression and vector interaction of a new form of BSBMV RNA-4 are described. Chapter 3 is dedicated to molecular and functional characterisation of BSBMV RNA-4 expressed protein.

Finally a general discussion and conclusion will describe results and scientific benefits obtained. I would also discuss future prospects to continue my work and increase knowledge on the virus transmission of BSBMV and BNYVV.

Chapter 1

Construction and analysis of genomic, full-length infectious clones of *Beet soil-borne mosaic virus* (BSBMV)

Introduction: viruses strategy

Depending of the host cell for their multiplication and protein expression, viruses minimize the size of their functional genome. During co-evolution with host plants, they developed many different strategies for efficient regulation and expression of their own proteins.

The majority of plant viruses possess single-stranded RNAs of positive polarity, so they can be directly used by the ribosomes for protein synthesis. On the other hand, negative-stranded single-stranded RNA viruses should be first transcribed to produce the messenger molecule for that region, minus-sense RNA genomes are therefore associated with the viral polymerase to produce infectious viral particles. Moreover there are ambisense RNA viruses, like the Tenuivirus *Rice stripe virus* (RSV), where proteins may be encoded by both genomic RNA and its complementary strand (Mandahar, 2006).

Initiation of protein synthesis in eukaryotes occurs mainly at the 5'-end of an mRNA molecule and ribosomes generally find the start codon with linear scanning mechanism (Kozak, 1986, 1999). Translation is then initiated at the AUG start codon within the best context and terminates when encountering a UAA, UAG or UGA stop codon (Caskey et al., 1968; Kozak, 1986). In plants, the most frequent context is AAACA**AUG**GC (Fütterer and Hohn, 1996), which is related to the mammalian sequence GCCRCC**AUG**G (Kozak, 1999): the optimal translation initiation motif includes a purine (R) at position -3 and the G at position +4. During evolving mechanisms, many viruses have developed several strategies, to express their polycistronic mRNAs that represent exceptions to the first AUG rule.

In general four levels of gene regulation expression can occur:

- genome segmentation;
- transcriptional regulation;
- translational regulation;
- post-translational regulation.

1.1 Regulation of gene expression through the genome segmentation

Many viruses split they genome in multiple DNAs or RNAs fragments (Mandahar, 2006). Fragmentation of the viral genome has two major advantages:

- Each genome segment can contain one or more ORFs but, usually, the coding sequences are more accessible for the translation by ribosomes;
- Multipartite genomes make finer the control of expression for genes which coding sequences are separated on different genomic segments.

As presented in the general introduction, Benyviruse (BNYVV and BSBMV) have multipartite genome composed by four to five RNAs.

1.2 Regulation of gene expression at the transcriptional level

Viruses may specifically express one or another protein from a genomic molecule by controlling the messenger RNAs (mRNAs):

- **Splicing** of viral mRNAs generates new coding sequences in the mRNA by removing parts of it. This kind of regulation is well described for plant viruses with DNA genome. Splicing is essential for the replication of *Wheat dwarf virus* (WDV) (Schalk et al., 1989) and for infectivity of *Cauliflower mosaic virus* (CaMV) (Kiss-Laszlo et al., 1995).
- **Sub-genomic (sg) RNAs**. A large number of RNA viruses, whether of positive or negative polarity, can produce one or more sgRNAs species that derive from the genome by internal initiation of RNA synthesis on the complementary gRNA strand. SgRNAs correspond to the mRNAs of the 3' proximal genes on polycistronic viral RNAs and they make possible the translation of coding sequences downstream the 5' proximal ORFs that are not required for replication. This strategy is widely used by plant viruses and allows, for instance, the expression of TGB proteins of BNYVV (Gilmer et al., 1992) and, putatively, BSBMV.

1.3 Regulation of gene expression at the translational level

During the initiation step of plant viruses translation, trans-regulation can occur with viral encoded proteins that specifically enhance translation of a downstream ORF in a bicistronic or a polycistronic mRNA as reported for several Caulimoviruses.

During translation initiation **leaky scanning** allows the expression of proteins from ORFs in the case of polycistronic mRNAs:

- Two ORFs can be consecutive on the same mRNA, so termination of the translation of the 5' proximal ORF is followed by a re-initiation at the second ORF, presumably without the release of the 40S ribosomal subunit.
- Two ORFs with in-frame initiation codons. The second start codon is preferred to the first one that is bypassed by ribosomes because the latter is placed in a suboptimal context different from the aforementioned AACA**AUG**GC (in plants) or is a non-AUG alternative start codon ((Kozak, 1989); (Ryabova et al., 2006)).
- Overlapping ORFs are the most frequent mechanism where the AUG codon of the second ORF overlaps the UGA termination codon of the first ORF. TGB are a group of three proteins whose genes generally overlap on the genome and leaky scanning is proposed to allow the expression of the third gene from TGB (TGBp3) of plant viruses like BNYVV (Zhou and Jackson, 1996) (Verchot et al., 1998) (Gilmer et al., 1992).

In some cases, "jumping" of the ribosomal machinery, allows ribosomes to "ignore" particular regions within the leader sequence of an mRNA that can contain short ORFs and encode just the downstream longer ORF. This mechanism is referred as **shunting** and transfers the ribosome from a donor to an acceptor site on the same mRNA, without involvement of mRNA scanning between these two sites. This mechanism is favoured by a strong RNA secondary structure on the leader sequence, as for CaMV (Futterer et al., 1990). However, no plus-sense RNA virus has yet been reported to use such translation strategy (Mandahar, 2006).

During the elongation step of translation, viruses can use a **frameshift** strategy to synthetize two proteins that are identical in their N-terminal region up to the frameshift point, but differ in their C-terminal region. This strategy is the result of ribosomes switch by one nucleotide towards the 5' (-1 nt) or 3' (+1 nt) extremity of mRNA. Essential signals in the RNA for frameshift are represented by an heptanucleotide (called slippery) sequence where frameshift occurs, separated by four to nine nucleotides from a downstream hairpin structure (Drugeon et al., 1999).

At the level of termination, regulation can occur by **readthrough** strategy where leaky terminators are recognized by tRNAs that add another amino acid instead of stopping translation and continue through next triplets to the final termination codon. This strategy is reported for many plant RNA viruses (Maia et al., 1996) and enables the expression of the BNYVV CP-RT proteins (Ziegler-Graff et al., 1985).

1.4 Regulation of gene expression at the post-translational level

Proteolytic cleavage strategy can produce separate structural and non-structural proteins from a viral polyprotein. This strategy is well documented for the polyprotein of Potyviruses where three distinct proteinases, P1, HC-Pro and NIa can release nine different proteins from a single polyprotein as for *Tobacco etch virus* (TEV) (Allison et al., 1985) or for BNYVV replicase (Hehn et al., 1997).

1.5 Reverse genetic approach to study viral protein expression

The term reverse genetics in virology refers to the use of recombinant DNA technology to convert viral genomes into (cDNA) and generate viruses from the cloned DNA. Since manipulating RNA genomes still remains cumbersome, genetic manipulation of RNA viruses has exclusively relied upon cDNA intermediates from which biologically active RNA molecules were generated. Usually for RNA viruses, full-length cDNA copies of each viral genomic RNA are flanked at 5' end with a transcription promoter (e.g. T7, T3 or SP6) and appropriated restriction enzyme at the extremity that allows, after RT-PCR, cloning in bacterial plasmid. Viral RNAs may be produced *in vitro* from these constructs by using the appropriate RNA polymerase. Biologically active transcripts produced *in vitro* RNAs from full-length cDNA clones with bacterial phage promoters have been first time reported for poliovirus (Racaniello and Baltimore, 1981). *In vitro* transcription was successfully employed to produce infectious transcripts from cloned cDNA of several plant viruses: *Brome mosaic virus* (BMV) (Ahlquist and Janda, 1984), *Tobacco mosaic virus* (TMV) (Dawson et al., 1986), *Beet necrotic yellow vein virus* (BNYVV) (Quillet et al., 1989) and many others.

Reverse genetics has now entered in the virologist custom to study viral cycles for which full-length infectious clones constitute a very powerful tool. Benefits of such approach are manifold, in particular:

- Working with single viral transcripts from constructs can guarantee single-virus infection and then avoid frequent mixed-infections that occur with soil-borne beet's viruses as BNYVV, BSBMV, BSBV and/or BVQ (Ratti et al., 2005);
- Function of single RNA and/or proteins can be investigate to understand pathogenicity mechanism and biological functions of the virus using different kinds of plants (*C. quinoa, B. macrocarpa*, *T. expansa* and *B. vulgaris*).

The use of reverse genetics permitted the characterisation of proteins involved in BNYVV pathogenicity (Jupin et al., 1992), cell-to-cell movement (Gilmer et al., 1992), systemic movement (Lauber et al., 1998), transmission (Rahim et al., 2007) and BSBMV pathogenicity (Ratti et al., 2009).

For all these reasons we decided to produce full-length cDNA clones of all BSBMV genomic RNAs.

Full-length cDNA clones of BSBMV isolate MRM06

Beet soil-borne mosaic virus (BSBMV) is one of the member of the *Benyvirus* genus and consists of four single stranded positive RNAs with CAP at 5' and poly-A at 3' end, packaged into rod-shaped particles (Lee et al., 2001). Recently Ratti et al. (2009) characterized the RNA-3 of a new BSBMV isolate (MRM06) and demonstrated that it is replicated and encapsidated by the BNYVV replication machinery (BNYVV RNA-1 and -2, also called Stras12). The symptoms induced by BSBMV RNA-3, together with Stras12 helper strain, on *C. quinoa* leaves are more similar to the necrotic local lesion caused by BNYVV RNA-5 p26 that to severe chlorotic local lesions, or yellow spot symptoms, induced by BNYVV RNA-3 encoded p25. Moreover, comparing amino acid sequence the authors reported that BSBMV RNA-3 p29 is much closer to BNYVV RNA-5 p26 (43%) than to BNYVV RNA-3 p25 (23%). Alignment of full-length BNYVV and BSBMV RNA-3 sequence revealed nucleotide identity of 61% and a 20 nts sequence called "coremin" (5'-GUCCGAAGACGUUAAACUAC-3'), conserved between both RNAs and also present in BNYVV RNA-5 and BSBMV RNA-4. Kinetics of local and systemic symptoms appearance on *B. macrocarpa* plants inoculated by BNYVV Stras12 helper strain supplemented with BNYVV RNA-3 or BSBMV RNA-3 transcripts, showed less efficient and delayed systemic movement of Stras12 supplemented with BSBMV RNA-3, when compared with BSBMV wild type infection. Such a difference could be linked to the presence of two "coremin" motifs within BSBMV (RNA-3 and -4) and the high accumulation of BNYVV RNA-3 that could compensate the lack of a second coremin. (Ratti et al., 2009).

2.1 Synthesis of a full-length infectious cDNA clone of BSBMV RNA-1

Sugar beet plants were grown on soil infested with *P. betae* carrying BSBMV isolate kindly supplied by Marc Richard-Molard (ITB, Paris). Total RNAs were extracted from infected sugar beet root using Trizol reagent (Invitrogen, Carlsbad, CA) and then used to obtain all full-length infectious cDNA clone of BSBMV.

BSBMV RNA-1 full length cDNA was synthesized by ImProm-II Reverse Transcriptase system (Promega, Madison, CA) using olido(dT) primer and then amplified by PCR using Pfu Ultra II Fusion Hotstart Polymerase (Agilent Technologies Italia) with a sense primer (5'-AAA*GCGGCCGC*TAATACGACTCACTATAGAAATTCTTCCCATTCGCCATCATTGAATCGTT-3') containing a T7 RNA polymerase promoter (underlined) and an *Not*I restriction site (*italic*), coupled with the oligo(dT) reverse primer (5'-AAA*ACGCGT*$(T)_{25}$-3') containing *Mlu*I site (*italic*). Primers match the extremities of the only BSBMV RNA-1 reference sequence to date published on GenBank database (NC_003506). PCR product was digested by *Not*I and *Mlu*I restriction enzymes, gel purified using Wizard SV Gel and PCR clean-up system (Promega, Madison, CA) and then cloned into *Not*I – *Mlu*I digested pUC19 (Fermentas) to obtain LB106 clone which was transferred into the *Escherichia coli* strain JM109. Ligation was performed according to Rapid DNA Ligation Kit (Fermentas).
LB106 and full-length infectious BNYVV RNA-2 type B clone, called pB2-14 (Quillet et al., 1989) were linearized with *Mlu*I and *Sal*I, respectively, before run-off transcription with the RiboMAX large-scale RNA production system T7 (Promega, Madison CA) in the presence of Cap analogue $m^7G(5')ppp(5')G$. Equal amounts of the transcripts were used to rub-inoculate *C. quinoa* leaves. As previously reported, chlorotic spots appeared 7 days post inoculation (dpi) on *C. quinoa* leaves rub-inoculated with BNYVV RNA-1 and -2 (Stras12). The appearance of local lesions indicated effective replication and cell-to-cell movement of the viral RNA-1 and -2. *C. quinoa* inoculated with LB106 and BNYVV RNA-2 transcripts didn't show symptoms even after 14 dpi.
Nucleotide sequences at 5' and 3' ends of LB106 were analysed looking for mutation putatively responsible for non-infectivity of full length BSBMV RNA-1 cDNA clone. Total RNA extraction from BSBMV-infected sugar beet roots was used to amplify two different amplicons performing RT-PCR reactions using primer pairs reported on Table I.1:

- Amplicon A (5' terminal zone) was digested by *Not*I and *Xba*I restriction enzymes, then gel purified and cloned in *Not*I – *Xba*I digested pUC19 to obtain LB126 clone.

- Amplicon B (3' terminal zone) was gel purified and inserted into the pGEM-T vector (Promega) to obtain clone LB153.

5' and 3' end cloning of BSBMV RNA-1

PCR Amplicon	Primers	Length (bp)	Restriction Enzyme	Clone
Amplicon A	BSBMV1 Not T7 F BSBMV1 R10	1,334	*Not*I - *Xba*I	LB126
Amplicon B	BSBMV1 F11 OligodT Mlu R	859	*Spe*I - *Mlu*I	LB153

Primers 5' Race on BSBMV RNA-1

Name	Sequence	Use
BSBMV1 R 294-275	5'-AGATCATGCTTCCAAATGGC-3'	RT
BSBMV1 R 195-176	5'-CGAAACAGCAAAACCTCCAT-3'	5' Race PCR

	Primers	Length (bp)	Restriction Enzyme	Clone
Amplicon C	BSBMV1 Not T7 5'Race F BSBMV1 R10	1,342	*Not*I - *Xba*I	EUB02

Table I.1 BSBMV RNA-1 primer: 5' and 3' end cloning or 5' Race pairs for obtain full

Sequences of clones were determined and compared with LB106 sequence. Three nucleotide substitutions were found in the ORF1 coding sequence ($G^{987}A$, $T^{6447}C$ and $T^{6450}C$) and one was found in the 3' UTR ($G^{6521}A$). LB126 was digested by *Not*I and *Xba*I and cloned in *Not*I-*Xba*I-digested LB106 to obtain LB128 where 5' terminal zone of BSBMV RNA-1 was replaced. Moreover LB153 was digested by *Spe*I and *Mlu*I and cloned in *Spe*I-*Mlu*I-digested LB128 to obtain LB158 where both 5' and 3' terminal zones of BSBMV RNA-1 were replaced.

No symptoms were detected 14 dpi on *C. quinoa* leaves rub-inoculated with transcripts obtained from LB158 and pB2-14 cDNA clones.

RNA extracts from infected BSBMV sugar beet roots was used to perform BSBMV RNA-1 5' RACE characterization according to a method adapted from (Bensing et al., 1996) using primers reported on Table I.1. Nucleotide sequence of BSBMV RNA-1 obtained by 5' RACE PCR showed 5 extra nucleotides after 6th nucleotide ^{6}GATCT^{11} and three nucleotide substitution ($T^{18}C$, $T^{19}C$ and $G^{21}A$), when compared with LB158 or published BSBMV RNA1 sequence (NC_003506). According to our 5' RACE results, new BSBMV RNA-1 5'-end primer was designed (BSBMV1 Not T7 5'Race F – 5'-AAA*GCGGCCGC*TAATACG-ACTCACTATAGAAATTC**GATCT**TTCCCA**CCC**A**C**CATCATTG-3') and amplicon C was obtained after RT-PCR reaction. Amplicon C was digested with *Not*I and *Xba*I restriction

enzyme and cloned in *Not*I - *Xba*I digested LB158 cDNA clone to obtain a new full length BSBMV RNA-1 cDNA clone (pUC17) with 6,680 nucleotides in length excluding the polyA. Mixture of the *in vitro* transcripts produced from plasmids pUC17 (tUC17) and pB2-14 (tBS2-14) were rub inoculated on *C. quinoa* leaves and chlorotic spots appeared 7 dpi confirming that pUC17clone is a full length infectious cDNA clone of BSBMV RNA-1 that is able to trans-replicate BNYVV RNA-2.

Northen blot analyses were performed on chlorotic lesions from *C. quinoa* leaves inoculated with tUC17 + tBS2-14 and Stras12. Total RNAs were extracted with Trizol reagent RNA extraction (Invitrogen, San Diego, CA) and encapsidated RNAs were either obtained using Protocol TM (Jupin et al., 1990). Membranes were probed with ^{32}P-labeled riboprobes specifically complementary to RNA-1 and -2 of BNYVV and BSBMV as reported in the appendix. Results demonstrated that transcripts obtained from BSBMV RNA-1 cDNA are fully functional as they insure the replication of BNYVV RNA-2 and are as well encapsidated.

Western blot analysis was performed on chlorotic lesions appeared on *C. quinoa* leaves inoculated with tUC17 + tBS2-14 and Stras12 using rabbit anti-BNYVV CP, as described in the appendix, raised against the BNYVV coat proteins. As showed in Fig. I.1 BNYVV CP is specifically detected in both sample, confirming encapsidation of BSBMV RNA-1 by BNYVV Coat Protein.

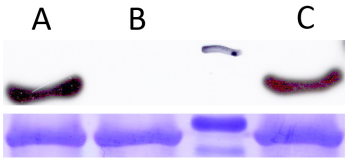


Fig. I.1 Western Blot analysis with anti-BNYVV CP of different inoculated *C. quinoa* leaves spots: (A) Stras12 isolate; (B) Mock; (C) tUC17 + tBS2-14.

2.2 Synthesis of a full-length infectious cDNA clone of BSBMV RNA 2

Full length BSBMV RNA-2 has been obtained from three PCR amplicons performed from the same cDNA using primer pairs reported in Table I.2. PCR amplicons were cloned separately on pUC19 vector, after cleavage with the appropriated restriction enzyme:

- *Not*I – *Xma*I for amplicon D to obtain clone LB5
- *Xma*I – *Xba*I for amplicon E to obtain clone LB7
- *XbaI* – *Bgl*II for amplicon F to obtain clone LB9

Full length BSBMV RNA-2 cDNA

PCR Amplicon	Primers	Length (bp)	Restriction Enzyme	Clone
Amplicon D (5' zone)	BSBMV2 NotI T7 F BSBMV2 XmaI R	1,843	*Not*I - *Xma*I	LB5
Amplicon E (middle zone)	BSBMV2 XmaI F BSBMV2 XbaI R	1,492	*Xma*I - *Xba*I	LB7
Amplicon F (3' zone)	BSBMV2 XbaI F OligodT25 BglII R	1,371	*Xba*I - *Bgl*II	LB9

Table I.2 BSBMV RNA-2 primer pairs used to obtain full-length cDNA clone.

After sub-cloning as described on Fig. I.2 we obtained a full length BSBMV RNA-2 cDNA clone, called LB38. This clone produced non infectious RNAs after *in vitro* transcription and co-inoculation with *in vitro* transcripts produced from pB15, the full-length infectious clone of BNYVV RNA-1 type B (Quillet et al., 1989).

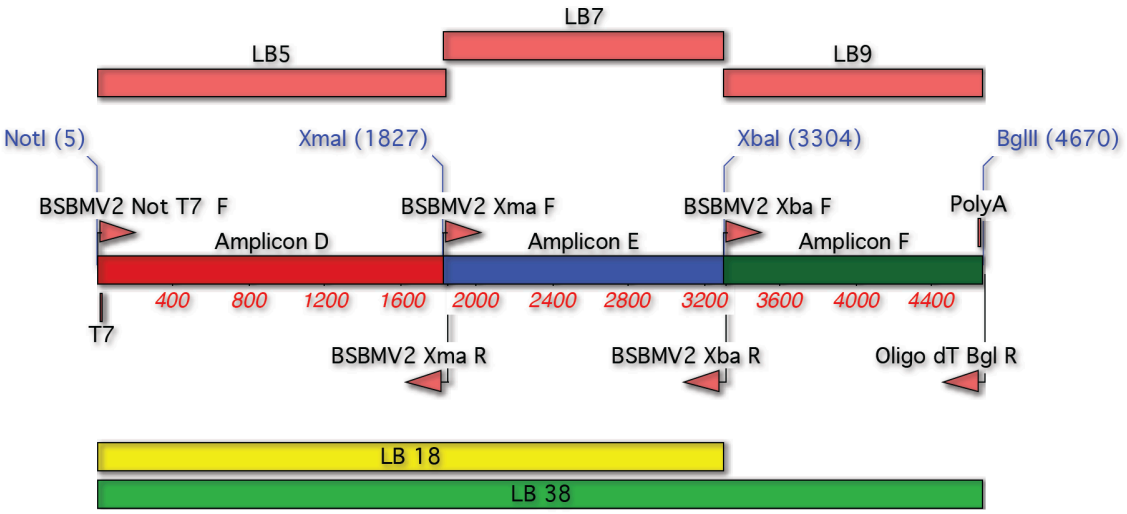


Fig. I.2 Schematic representation of BSBMV RNA-2 full length cDNA cloning. After PCR reactions using specific primers described in Table I.2, D, E and F amplicons were cloned into different pUC19 vector using *Not*I-*Xma*I, *Xma*I-*Xba*I and *Xba*I-*Bgl*II restriction enzymes obtaining respectively LB5, LB7 and LB9 plasmids. Amplicon E, extracted from LB7 clone using *Xma*I and *Xba*I restriction enzymes, has been inserted into LB5 clone within *Xma*I and *Xba*I sites, obtaining LB18. Finally into *Xba*I-*Bgl*II-digested LB18 has been ligated *Xba*I-*Bgl*II-digested Amplicon F (extracted from LB9 clone) to obtain full length BSBMV RNA-2 on LB38 clone.

As previously reported for BSBMV RNA-1 we decided to investigate the correct nucleotide sequence at 5' and 3' ends. Different RT-PCR amplicons were obtained using primer pairs reported on Table I.3:

- Amplicon G was cloned into pGEM-T vector to obtain LB164
- Amplicon H was cloned into pGEM-T vector to obtain LB156

5' and 3' end cloning of BSBMV RNA-2

PCR Amplicon	Primers	Length (bp)	Restriction Enzyme	Clone
Amplicon G (5' end)	BSBMV1 Nco F BSBMV2 Xma R	1,147	*Nco*I - *Xma*I	LB164
Amplicon H (3' end)	BSBMV2 F1 Oligo dT25 BglII R	1,414	*Xba*I - *Bgl*II	LB156

Primers 5' Race on BSBMV RNA-2

Name	Sequence	Use
BSBMV2 384-365	5'-TTGAGGGTAACTGGAAACCG-3'	RT
BSBMV2 293-274	5'-TAACAGGTAAGCTTGCGGCT-3'	5' Race PCR

	Primers	Length (bp)	Restriction Enzyme	Clone
Amplicon I (5' Race RT-PCR)	BSBMV2 Not T7 AA F BSBMV1 Nco R	681	*Not*I - *Xma*I	EUB17

Table I.3 BSBMV RNA-2 primer: 5' and 3' end cloning or 5' Race pairs used to obtain or characterize full-length cDNA clone.

Sequence analysis of LB156 and LB164 clones revealed several nucleotide substitutions when compared with LB38 sequence. Three nucleotide substitution were found in the ORF2 coding sequence ($C^{972}T$, $C^{1018}T$ and $G^{1080}A$), one was found in the ORF6 ($C^{4052}T$) and one in the 3'UTR ($C^{4613}T$) followed by insertion of two nucleotide ($^{4613}TG^{4615}$). LB156 was digested by *Xba*I and *Bgl*II and cloned in *Xba*I – *Bgl*II digested LB38 to obtain LB157. LB164 was then digested by *Nco*I and *Xma*I and cloned in *Nco*I – *Xma*I digested LB157 to obtain LB177.

Moreover, 5' RACE characterisation of BSBMV RNA-2 (see Table 3 for details) revealed that the first two GG nucleotides were incorrect and needed to be substituted by AA nts (G^1A and G^2A). We corrected the sequence by designing a new primer on 5' BSBMV RNA-2 and performing RT-PCR from which the amplicon I was obtained and then cloned into pGEM-T vector to obtain EUB17. 5' BSBMV RNA-2 was extracted from EUB17 by *Not*I - *Nco*I digestion and cloned into *Not*I-*Nco*I digested LB177 to obtain a new full length BSBMV RNA-2 cDNA clone (EUB22).

The three full-length cDNA constructs in pUC19 (LB38, LB177 and EUB22) were linearized with *Bgl*II then run-off transcribed. All transcripts were separately rub-inoculated with pB15's transcripts onto *C. quinoa* leaves but no symptoms were detected 14 dpi.

2.2.1 Are TGBs proteins fully functional?

As described by Guilley et al. (2009), Benyviruses need three main functions for successful multiplication on a plant host: (i) replication, provided by RNA-1 that encodes the viral RdRp; (ii) movement from the initial point of infection, provided by triple gene block (TGB) proteins expression; (iii) suppression of the host RNA silencing mechanism, provided by cysteine rich p14 protein. As RNA-2 is involved in the last two functions virus infection in *C. quinoa* leaves was successful obtained supplementing BNYVV RNA-1 with Rep3-P30, encoding the *Tobacco mosaic virus* (TMV) movement protein, and Rep5-P19 encoding the *Tomato bushy stunt virus* (TBSV) silencing suppressor protein (Guilley et al., 2009).

In order to verify TGB and p14 proteins functionality of EUB22 clone its transcript has been supplemented by pB15 and Rep3-30 transcripts and rub-inoculated together on *C. quinoa* as described before. Chlorotic spots were observed on inoculated leaves 7 d.p.i. This result suggests that EUB22 is replicated by BNYVV RdRp and that its p14 protein is correctly expressed and full functional as observed chlorotic spots were identical to those induced by Stras12 isolate and no small necrotic local lesions as those induced by BNYVV RNA-1 and -2 carrying a non functional p14 protein (Gilmer et al., 1992). The most important information obtained from this results was that TMV p30 protein expression assisted cell-to-cell movement of the chimeric virus BNYVV RNA-1 and BSBMV RNA-2, suggesting that one, two or all TGB proteins carried by EUB22 clone were not functional.

Bleykasten-Grosshans et al. (1997) described three cDNA clones: Rep42, Rep1315 and Rep15 that can express, by BNYVV RNA 3-derived replicon (Jupin et al., 1990), first (TGBp1), second plus third (TGBp2 and TGBp3) or third (TGBp3) BNYVV's TGB proteins, respectively. Transcripts of pB15 (tBS15) and EUB22 (tUC22) clones have been rub-inoculated on *C. quinoa* together with transcripts obtained from Rep42, Rep1315 or Rep15 clones as described in Table I.4. As *C. quinoa* symptoms analysis (yellow spots appearance) confirms complementation of movement proteins just using Rep3 p13-p15 clone (Table I.4) we concluded that some problems occur, maybe on nucleotide sequence, in TGBp2 and TGBp3 of EUB22 clone and that TGBp1 of the same clone is fully functional.

Inoculum	Symptoms (7 dpi)
Stras12	YS
tBS15 + tEUB22	---
tBS15 + tEUB22 + tRep42	---
tBS15 + tEUB22 + tRep1315	YS
tBS15 + tEUB22 + tRep15	---

Table I.4 Mechanical inoculation of *C. quinoa* plant, local symptoms after inoculation of BNYVV Stras12 helper strain or BNYVV RNA-1 transcripts (tBS15) and BSBMV RNA-2 transcripts (tEUB22) with three TGB proteins express in different viral replicon. The appearance of yellow spots (YS) indicates the correct replication and cell-to-cell movement of the virus.

cDNA from total RNA extraction from infective BSBMV sugar beet roots was used to performed PCR on BSBMV TGBp2 and TGBp3 using primer sense BSBMV p13 F (5'-AAA*CCATGG*ATGTCTAGAGAAATAAC-3', *Nco*I site in *italic*) and primer reverse BSBMV p15 R (5'-AAA*GGATCC*TTAACTATGATACCAAAAC-3', *Bam*HI site in *italic*) designed at 5' end of TGBp2 coding sequence and 3' end of TGBp3 coding sequence, respectively. PCR amplicons were digested with *Nco*I and *Bam*HI and cloned into the BNYVV RNA-3 derived replicon (Rep3), and six different Rep3 BS p13-p15 cDNA clones (from I to VI) were selected during cloning procedure. Only Rep3 BS p13-p15 cDNA clone (IV) induced yellow spots symptoms 7 dpi when inoculated together with pB15 and EUB22 transcripts. Comparison of Rep3 BS p13-p15(IV) and EUB22 sequences permitted to identified one mutation on EUB22 p13 ORF ($G^{3362}A$) that induce amino acid substitution ($V^{31}M$) and one nucleotide deletion on EUB22 p15 ORF ($^{3975}T^{3977}$) which is responsible for the production of a 11 amino acids shorter p15 (13,1 kDa instead of 14,6 kDa).

PCR site-directed mutagenesis on EUB22 cDNA clones was performed using primer specifically designed on p13 ORF (reported on Table I.5). Such modified amplicon was used to replace a DNA fragment on EUB22 cDNA clone using restriction enzymes *Xba*I and

PCR side-directed mutagenesis

BSBMV p13 ORF			**BSBMV p15 ORF**		
First PCR			*First PCR*		
	Primers	bp		Primers	bp
5' - end	BSBMV2 Xma F BSBMV2 p13 Mut R	1,581	5' - end	BSBMV2 XbaI BSBMV2 p15 Mut R	750
3' - end	BSBMV2 p13 Mut F OligodT25 BglII R	1,293	3' - end	BSBMV2 p15 Mut F OligodT25 BglII R	678
Second PCR			*Second PCR*		
	Primers	bp		Primers	bp
Fusion	BSBMV2 XbaI Oligo dT25 BglII R	1,372	Fusion	BSBMV2 XbaI Oligo dT25 BglII R	1,373

Table I.5 PCR site-directed mutagenesis of p13 and p15 protein of BSBMV RNA-2.

*Bgl*II to obtain MD231 clone. A second PCR site-directed mutagenesis on MD231 cDNA clone was performed to correct nucleotide deletion on p15 ORF using specific primer (Table I.5) and then substitute the sequence, as described above, on MD231 cDNA clone to obtain pUC29.

The following rub-inoculations on *C. quinoa* leaves were performed, after in vitro transcription, as reported in Table I.6:

Inoculum	Symptoms (7 dpi)
Stras12	YS
tBS15 + tEUB22 + Rep1315	YS
tBS15 + tMD231 + tRep15	YS
tUC15 + tUC29	YS

Table I.6 Leaves symptoms 7 dpi on *C. quinoa* mechanical inoculated leaves combining BNYVV RNA-1 transcripts (tBS15) with BSBMV RNA-2 transcripts (tEUB22) and p13-p15 TGB proteins or tMD231 and p15 TGB proteins or full-length infectious BSBMV RNA-2 transcripts (tUC29).
YS = yellow spots

Yellows spots appeared 7 dpi on all samples, allowing us to conclude that pUC29 is an infectious full length cDNA clones of BSBMV RNA-2 that is able to be trans-replicated by BNYVV RNA-1. Northern Blot and Western Blot analysis of chlorotic lesion appeared on *C. quinoa* confirmed that BSBMV RNA-2 is correctly replicated by BNYVV RNA-1 and the coat protein (encoded by RNA-2) is able to encapsidate both RNAs (Fig. I.3).

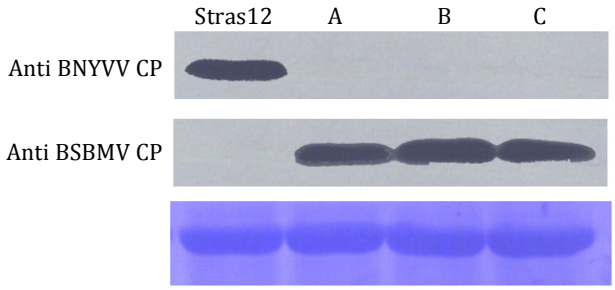


Fig. I.3 Western Blot analysis with anti-BNYVV CP and BSBMV CP of different inoculated *C. quinoa* leaves spots: (A) Stras12 isolate; (B) tBS15 + tEUB22 + Rep1315; (C) tBS15 + tMD231 + tRep15; (D) tBS15 + tUC29.

2.3 Synthesis of a full-length infectious cDNA clone of BSBMV RNA 4

Specific oligonucleotide primer, complementary to the 5' end of BSBMV RNA-4 sequence (NC_003508), was used for full length BSBMV RNA4 cDNA clone production. After total RNAs extraction from BSBMV infected sugar beet roots, RT reaction was performed with oligodT_{25} primer followed by High Fidelity PCR amplification using primer BSBMV RNA-4 T7 F (5'-AAA*GCGGCCGC*TAATACGACTCACTATAGaaattcaaaactcaaaaatataatttgtatttccagttg-3', *Not*I restriction enzyme site is *in italic* and T7 nucleotides promoter is underlined) and primer oligodT_{25} *Bgl*II R (5'-AAA*AGATCT*$(T)_{25}$-3', *Bgl*II site is in italic). PCR amplicon of full length BSBMV RNA-4 was then digested with *Not*I and *Bgl*II restriction enzyme and ligated into *Not*I-*Bgl*II digested pUC19 to obtain pUC47 clone.

Analysis of pUC47 nucleotide sequence revealed a 1,730 nts long BSBMV RNA-4, excluding the 3' poly(A) tail. pUC47 was linearized with *Bgl*II before run-off transcription as previous described obtaining the transcripts tUC47. tUC47 were then inoculated together with the BNYVV Stras12 helper strain on *C. quinoa* leaves and 7 dpi necrotic spots were observed confirming infectivity of full length BSBMV RNA-4 cDNA clone. Further experiments and results about BSBMV RNA-4 characterisation are described in Chapter 2 and 3.

BSBMV MRM06 isolate: description and BNYVV comparison

The nucleotide sequence and genomic organization of new isolate BSBMV MRM06 were determined and compared to BSBMV EA isolate described by Lee et al. (2001).

BSBMV RNA-1 MRM06 isolate is 6,679 nt in length (accession number JF513082), excluding the 3' poly(A) tail, and contains a single ORF which encode a 238 kDa polypeptide with a putative MTR/HEL and RNA dependent RNA polymerases (RdRp) located in the N- and C- terminal parts, respectively. MRM06 RNA-1 shares 99.4% nucleotide identity with EA RNA-1, in particular, some nucleotide differences have been evidenced on MRM06 RNA-1 5' end (5'-AAATTC**GATCT**TTCCCACCCACC-3', where insertions are indicated in bold and mutations are underlined) that resulted essential for infectivity of the full length cDNA clone. Comparing MRM06 and EA amino acid sequence, 47 amino acids substitution are located in the MTR domain (major part between aa 110-190) liable of 96,6% identity and just 2 amino acids exchanges are located in the RdRp domain ($E^{1824}K$ and $I^{2058}M$) liable of 99,7% identity. Sequence motifs of a type 1 putative MTR domain have been identified at amino acid positions 218-228, 271-278 and 407-414 on MRM06 isolate according to amino acid positions 220-230, 273-280, and 407-420 reported for EA isolate by Lee (2001). The NTP-binding helicase motif (Gly-X-X-Gly-X-Gly-Lys-Ser, where X represents any amino acid), that is predicted to be involved in duplex unwinding during viral RNA replication and translation, is located at amino acid positions 944-951 or 941-948 on isolate EA or MRM06, respectively. Moreover, the motif GDD (Gly-Asp-Asp), characteristic of RdRp is located at amino acid positions 1939-1941 or 1936-

1938 of isolate EA or MRM06, respectively. Finally, all the previous described sequence motifs are conserved between RNAs-1 of BSBMV and BNYVV type A, B or P. BSBMV RNA-1 MRM06 isolate and BNYVV RNA-1 share 77% nucleotides sequence identity leading to 84% and 87,5% on amino acids identity for MTR/HEL and RdRp domains, respectively. Amino acids identity and higher amino acids similarity (around 92 %) between BSBMV – MRM06 and BNYVV RNAs-1 ORFs are in accordance with capability of BSBMV RNA-1 to replicate BNYVV RNA-2. This result demonstrates the complementarity and the identical functions of polypeptides encoded by BSBMV and BNYVV RNA-1.

BSBMV RNA-2 MRM06 isolate is 4,615 nts in length (accession number JF513083), excluding the 3' poly(A) tail, and contains six ORFs that encode proteins involved in different functions: viral Capsid Protein (CP), Read-through (RT), Triple Gene Block protein (TGBp1, TGBp2 and TGBp3) and a cysteine-rich (Cys-R) protein. High nucleotide identity (99,8%) has been obtained comparing MRM06 and EA RNA-2. MRM06 isolate showed 4 nucleotide substitutions ($A^{1}G$, $A^{2}G$, $G^{99}C$, $G^{119}C$) and one deletion ($^{93}G^{95}$) in the 5' UTR, 2 nucleotide substitution on RT protein ($A^{1567}G$ and $A^{2000}T$) translated, respectively, to $M^{475}V$ and $Q^{617}L$ amino acid mutation and one nucleotide substitution on Cys-R protein ($C^{4272}T$) which leads to a silent mutation. Amino acids sequence between BSBMV isolates MRM06 and EA is highly conserved within five proteins (CP, TGB proteins and Cys-R) with only two changes on RT protein. As reported in Table I.7 comparison between MRM06 and BNYVV RNA-2 from type A, B and P showed high identity score within Triple Gene Block proteins (TGBp1: 74.7 - 75%; TGBp2: 81.4 – 82.2%; TGBp3: 63.6 – 65.2%), medium identity score between CP (56 – 57.6%) and RT (58.3 – 58.7%) proteins and low identity score on Cys-R protein (32.6 – 34.6%). Similitude between proteins encoded by BSBMV and BNYVV RNAs-2 increase in terms of amino acid similarity suggesting a common function of each corresponding ORF product. Moreover, according to our results we can speculate about complementation of TGBp2 and TGBp3 proteins (p13 and p15 respectively) between BNYVV and BSBMV on cell-to-cell movement function. In other words, as BNYVV p13 and p15 proteins, expressed by viral vectors, are able to restore a defective cell-to-cell movement function of mutated, then not functional, BSBMV p13 and p15 proteins, we can conclude that TGBp2 and TGBp3 proteins from BNYVV and BSBMV clearly exert the same function during virus infection.

As previously reported by Ratti et al. (2009) BSBMV RNA-3 MRM06 isolate is 1,720 nts in length (accession number EU410955) and contains one ORF that encode a 29 kDa protein

(p29) involved on virus symptoms. In this case, only 4 nts substitutions were detected when the sequence was compared with the RNA-3 sequence of the EA BSBMV isolate described by Lee (NC_003507): one substitution was found in the 5' UTR ($G^{355}A$) and three along the p29 coding sequence ($T^{808}A$, $A^{887}G$ and $T^{1000}C$) which leads, respectively, to $D^{127}E$, $N^{154}D$ and a silent mutation that determine amino acid identity score of 99,2%. As shown in Table 9 BSBMV RNA-3 encoded p29 share around 23% of amino acid sequence identity with the 25 kDa ORF of BNYVV RNA-3.

BSBMV RNA-4 MRM06 isolate is 1,730 nts in length (accession number FJ424610) and show an high identity nucleotide score (99,4%) with RNA-4 described by Lee (NC_003508) between nucleotides 1-608 and 1,138-1,730 where we identified one nucleotide substitution ($T^{219}C$) and two nucleotides deletion ($^{269}C^{270}$ and $^{296}C^{297}$) on 5' UTR, one nucleotide substitution on ORF1 ($C^{521}T$) leading to silent mutation and three nucleotide substitutions on 3'UTR ($T^{1572}C$, $T^{1575}C$ and $G^{1594}T$). Lee et al. (2001) identified one ORF encoding a protein with predicted mass of 13 kDa that show 20% amino acid identity with p31 (31 kDa protein encoded by BNYVV RNA-4) increasing to 42% amino acid identity if just the N-terminal half of p31 is considered. However, the RNA-4 of MRM06 isolate shows 529 additionally nucleotides between positions 608-1,138, that determine a new BSBMV RNA-4 ORFs organization: two putative ORFs have been identified, the first one (nucleotides 383 – 1,234) encode a protein with predicted mass of 32 kDa (ORF1; p32) and the second one (nucleotides 885 – 1,244) express an expected product of 13 kDa (ORF2; p13). BSBMV RNA-4 p32 shows 49% amino acid identity and 68% amino acid similarity with p31 sequence.

In the Table I.7, we reported nucleotide and amino acid sequence identity analyses of the viral proteins of BSBMV MRM06 isolate compared with BSBMV EA isolate, BNYVV type A, B and P and the *Rice stripe necrosis virus* (RSNV), a candidate member of *Benyvirus* genus (Lozano and Morales, 2009).

Table I.7 Comparative nucleotide (a) and amino acid (b) sequence identity analyses of the viral proteins of *Beet soil-borne mosaic virus* (BSBMV) isolate MRM06 and *Benyvirus* viruses[a,b]

(a)	**Nucleotide identity score**			
BSBMV – (MRM06)	RNA1 (****)	RNA2 (***)	RNA3 (****)	RNA4 (****)
BSBMV – (EA)	99,4 %	99,8 %	99,8 %	69,1 %
BNYVV – A type	77,0 %	66,5 %	55,0 %	56,1 %
BNYVV – B type	72,8 %	66,4 %	55,4 %	47 %
BNYVV – P type	77,0 %	66,6 %	55,2 %	55,7 %
RSNV	53,8 %	45,2 %	na	na

(b)	**Amino acid identity (and similarity) score**									
BSBMV – (MRM06)	**RNA1 238 kDa**		**RNA2**						**RNA3**	**RNA4**
	MeT/H[c]	**RdRp[c]**	**CP (21 kDa)**	**RT (75 kDa)**	**TGBp1 (42 kDa)**	**TGBp2 (13 kDa)**	**TGBp3 (15 kDa)**	**Cys-R (14 kDa)**	**29 kDa (ORF1)**	**32 kDa (ORF1)**
BSBMV – (EA)	96,6 % (97,2 %)	99,7 % (99,8 %)	100 %	99,7 % (99,9 %)	100 %	100 %	100 %	100 %	99,2 % (99,6 %)	31,4 % (33,6 %)
BNYVV – A type	84,3 % (92,4 %)	87,5 % (92,8 %)	57,1 % (73,3 %)	58,7 % (72,1 %)	75 % (88,5 %)	82,2 % (89 %)	65,2 % (75,0 %)	32,6 % (49,6 %)	22,4 % (36,7 %)	49,8 % (67,7 %)
BNYVV – B type	85,0 % (92,9 %)	87,6 % (92,7 %)	57,6 % (72,8 %)	58,3 % (71,7 %)	75 % (88,5 %)	81,4 % (88,1 %)	65,2 % (75,0 %)	34,6 % (50,4 %)	22,8 % (35,9 %)	49,8 % (68,4 %)
BNYVV – P type	84,7 % (92,7 %)	87,3 % (92,8 %)	56 % (73,3 %)	58,7 % (72,2 %)	74,7 % (88,5 %)	82,2 % (89 %)	63,6 % (75,0 %)	32,6 % (49,6 %)	22,0 % (35,5 %)	49,5 % (68,1 %)
RSNV	37,5 % (55,3 %)	55,4 % (70,5 %)	29,8 % (44 %)	20,7 % (34,2 %)	34,7 % (52,5 %)	41,5 % (56,9 %)	24,1 % (44,5 %)	15,6 % (29,9 %)	na[d]	na

Data shown are expressed as percentages of nucleotide or amino acid identity and amino acid similarity in brackets.

[a]Using MacVector version 11.1

[b] The sequence of Benyviruses used for these analyses are available in the Genbank under the following accession numbers: *Beet soil-borne mosaic virus* (BSBMV), isolate MRM06 (JF513082, JF513083, EU410955, FJ424610) isolate EA (NC_003506, NC_003503, NC_003507, NC_003508); *Beet necrotic yellow vein virus* (BNYVV) A type (D84410, D84411, D84412, D84413), B type (X05147, X04197, M36894, M36897), P type (HM126464, HM117903, DQ682454, DQ682453); *Rice stripe necrosis virus* (EU099844, EU099845).

[c] The predicted ORF on RNA1 was divided on N-terminal 1500 amino acids and C-terminal 600 amino acids to compare the methyltransferase/helicase (Met/H) and RNA-dependent RNA polymerase (RdRp) regions, respectively. CP, coat protein; RT, read-through; TGB, triple gene block; Cys-R, cysteine-rich

[d]Not applicable or not present

Chapter 2

Beet soil-borne mosaic virus RNA-4 encodes a 32 kDa protein essential for virus transmission through *Polymyxa betae*

Beet soil-borne mosaic virus RNA-4 encodes a 32 kDa protein essential for virus transmission through *Polymyxa betae*

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Abstract

Beet soil-borne mosaic virus (BSBMV) together with *Beet necrotic yellow vein virus* (BNYVV) are members of the *Benyvirus* genus, transmitted by *Polymyxa betae.* They possess a similar genomic organization: RNAs -1 and -2 are essential for infection and replication while RNAs -3 and -4 play important roles on disease development and vector-mediated infection in sugar beet roots. We molecularly and functionally characterized a new 1,730 nucleotides long form of BSBMV RNA-4 that could encode for 32kDa (p32) and 13kDa products. We demonstrated that BSBMV RNA-4 is amplified by BNYVV viral machinery *in planta* and that it can substitute BNYVV RNA-4 for an efficient transmission through the vector *P. betae* in *Beta vulgaris* plants. Analysis of BSBMV RNA-4 encoded protein evidence possible post translation modification site or its strong interaction with host protein(s). BSBMV p32 protein expressed by BNYVV RNA-5-based viral vector (Rep5) demonstrated that the p32 protein itself and not full-length BSBMV RNA-4 is essential for the viral transmission.

Keywords: Benyvirus, *Polymyxa betae*, trans-replication, transmission

Introduction

Beet soil borne mosaic virus (BSBMV) is a member of *Benyvirus* genus together with *Beet necrotic yellow vein virus* (BNYVV), the causal agent of Rhizomania disease, which induce abnormal rootlet proliferation and is widespread in the sugar beet growing area in Europe, Asia and America (Peltier et al., 2008). *Rice stripe necrosis virus* (RSNV) has been also recently proposed as new member of *Benyvirus* genus (Lozano and Morales, 2009).

In nature BSBMV and BNYVV are vectored by the plasmodiophorid *Polymyxa betae* and have similar host range, particles number and morphology. BSBMV is widely distributed only in the United States and, up to date, it has not been reported in others countries. It was first identified in Texas in 1988 as a sugar beet virus morphologically similar but serologically distinct from BNYVV (Heidel et al., 1997).

Subsequent sequence analysis of BSBMV RNAs evidenced similar genomic organization whit BNYVV but sufficient molecular differences to distinct BSBMV and BNYVV into two different species (Lee et al., 2001); (Rush, 2003).

Benyviruses field isolates usually consist of four RNA species but some BNYVV isolates contain a fifth RNA. RNAs -1 contain a single long ORF encoding polypeptide that shares amino acid homology with known viral RNA-dependent RNA polymerases (RdRp), methyltransferase (MTR) and helicases (HEL). RNAs 2 contain six ORFs, the first one, capsid protein, is located at the 5'-terminus followed by a leaky UAG stop codon, whose suppression leads to the expression of the read-through translation protein (RT) involved on virus transmission. Next 3 ORFs, triple gene block proteins (TGB), are required for cell-to-cell virus movement. The sixth 14 kDa ORF is a cysteine-rich protein with post-translation gene silencing suppressor activity (Dunoyer et al., 2002). RNAs -3 encode a single protein with predicted mass of 25 kDa for BNYVV and 29 kDa for BSBMV which shares 23% amino acid sequence identity. RNAs -3 are involved on disease symptoms and are essential for virus systemic movement. BNYVV RNA-4 encoded one 31 kDa protein (p31) that plays a multifunctional role in enhanced symptom expression, root-specific silencing suppression, efficient vector interactions and virus transmission by *P. betae* (Rahim et al., 2007).

Studies performed on a 1,203 nts long form of BSBMV RNA-4 described a single putative ORF encoding for a protein, with a predicted mass of 13 kDa, considerably smaller than p31 encoded by BNYVV RNA-4 (Lee et al. (2001) .

Some BNYVV isolates contain a fifth RNA, encoding 26 kDa protein (p26) capable to

improve virus infections and accumulation in the hosts ((Heijbroek et al., 1999); (Link et al., 2005)). It has been recently reported that p29, encoded by BSBMV RNA-3, is much closer to the RNA-5-encoded p26 than to BNYVV RNA-3-encoded p25. Moreover it has been demonstrated that BSBMV RNA-3 can be trans-replicated and trans-encapsidated by the BNYVV helper strain (RNA-1 and -2) but such replication does not occur in the presence of BNYVV RNA-3 due a replication competition (Ratti et al., 2009), confirming the strong relation between the two viruses.

Studies of interaction between BNYVV and BSBMV infecting the same beet plants, through mechanical inoculation, showed a high degree of reciprocal cross-protection, a phenomen that usually occurs between virus strains, suggesting a close relation between the two species (Mahmood and Rush, 1999). However, the absence of immunological cross-reaction between the two viruses, and the susceptibility to BSBMV of plants resistant to BNYVV (Lee et al., 2001); (Wisler et al., 2003), indicated that the two viruses are distinct.

In our study, we reported a new form of BSBMV RNA-4. We confirmed the capability of BNYVV helper strain to replicate, encapsidate and spread BSBMV RNA-4 *in planta*. Using BNYVV helper strain, we also demonstrated that BSBMV RNA-4 could substitute BNYVV RNA-4 for an efficient transmission through the vector *P. betae* in *B. vulgaris* plants. However frame shift experiment on BSBMV RNA 4's ORFs and BSBMV p32 protein expression by BNYVV RNA-5-based viral vector (Rep5) demonstrated that just p32 protein expression is essential for an efficient transmission of the virus.

Materials and methods

2.1 Synthesis of a full-length infectious cDNA clone of BSBMV RNA 4

Sugar beet plants were grown on BSBMV infected soil kindly supplied by Marc Richard-Molard (ITB, Paris). Total RNAs were extracted from infected sugar beet roots using Trizol reagent (Invitrogen, Carlsbad, CA). RNA-4 cDNA was synthesized by Improm-II Reverse Transcriptase system (Promega, Madison, CA) using oligo(dT) primers and then amplified by PCR using Pfu Ultra II Fusion Hotstart Polymerase (Agilent Technologies). The forward primer BSBMV4 NotI T7 and reverse primer OLIGOdT25 BglII R were used. PCR products were digested by *Not*I and *Bgl*II and cloned in *Not*I-*Bgl*II-digested pUC19 (Fermentas) to obtain a full length cDNA clone, pUC47 (GenBank ID: FJ424610). Full-length pUC47,

linearized by *Bgl*II, was used to synthetize BSBMV RNA-4 infectious run-off transcripts, named tUC47. In a similar way BNYVV RNA-4 full-length cDNA was obtained by RT-PCR from BNYVV B-type infected sugar beet roots and cloned into pUC19 between *Not*I and *Bgl*II restriction sites to obtain pUC-4B clone.

Primer	Sequence (5' - 3')	Restriction enzyme
BSBMV4 NotI T7 F	AAA*GCGGCCGC*TAATACGACTCACTATAGAAATTCAAA ACTCAAAAATATAATTTTGTATTTCC	*Not*I
OLIGOdT25 BglII R	AAAA*GATCT*$T_{(25)}$	*Bgl*II
BSBMV Flag-p32 NcoI	AAA*CCATGG*ACTACAAGGACGACGACGACAAG**CCAGGA** GCCGATGTGGAGATTTGCCG	*Nco*I
BSBMV p32 BamHI	AAA*GGATCC*TCACTGAAAATCTTGTTCGAAACAAAAC	*Bam*HI
BSBMV Flag-p13 NcoI	AAA*CCATGG*ACTACAAGGACGACGACGACAAG**CCAGGA** TGGATCAGTATACCCTTCCCTC	*Nco*I
BSBMV p13 BamHI	AAA*GGATCC*TCACAAATAATCACTGAAAATCTTG	*Bam*HI
BNYVV FlagP31 XmaI F	AAA*CCCGGG*ATGGACTACAAGGACGACGACGACAAG**CC AGGA**GCTGATGGAGAGATATGTCGGTG	*Xma*I
BNYVV p31 SalI BamHI R	AAA*GTCGACGGATCC*CTAATCGTGATAAAAGACAAAC	*Sal*I and *Bam*HI
FS P32 F NcoI	AAA*CCATGG*ACTGGCCGAT*TGGAGATTTG	*Nco*I
FS P32 F1	TCACTATAGATGCTGGATCAG	---
FS P32 R1	TATACTGATCCAGCATCTATAGTG	---
FS P32 F2	CCCTCTCAATGAGAATGGTTGTGC	---
FS P32 R2	TCATTGAGAGGGAAGGGTATACTG	---
FS P32 F3	ACGATTTGTGTAACGTGGTTTGGTTGGAG	---
FS P32 R3	ACCACGTTACACAAATCGTTGAGTGG	---

Table II.1 **BNYVV and BSBMV RNA-4 primers**. Restriction enzyme sequences appear in italic, whereas, Flag and T7 promoter sequences are underlined and Gly and Pro amino acids codon are in bold. The Oligo(dT) tract is represented by $T_{(25)}$. Mutated nucleotides are in red and * is G deletion on p32 nucleotide sequence.

Putative ORF sequences encoding 32 kDa and 13 kDa proteins were amplified by PCR using a sense primer (BSBMV Flag-p32 NcoI for ORF1 and BSBMV Flag-p13 NcoI for ORF2, Table II.1), containing *Nco*I restriction site and a FLAG epitope sequence, and an antisense primer (BSBMV p32 BamHI and BSBMV p13 BamHI, respectively for ORF1 and ORF2) carrying a *Bam*HI site.

The PCR-amplified fragments were cloned into Rep3 viral vector (Bleykasten-Grosshans et al., 1997) using *Nco*I and *Bam*HI restriction enzyme obtaining Rep3-Flag-p32, Rep3-Flag-p13 and clones. Similarly, BNYVV FlagP31 XmaI F and BNYVV p31 SalI BamHI R primers pair (Table 1) were also used to amplify the BNYVV RNA-4 p31 sequence further cloned into Rep3 viral vector, between *Xma*I and *Bam*HI sites to obtain Rep5-Flag-p31 construction. A new set of sense primers, depleted of the FLAG nucleotide sequence from primers described above, were used to obtain Rep5-p32, Rep5-p13 Rep5-p31 clones carrying wild type p32, p13 and p31 proteins into Rep5 viral vector (Schmidlin et al., 2005), respectively. FLAG epitope was also inserted in frame within p32 sequence (between amino acids 202 and 203) in pUC47 to produce BSBMV RNA-4-FlagIN.

Frame shift of both BSBMV RNA-4's ORFs was obtained with PCR site directed mutagenesis modifying ORFs sequences by four nucleotides insertion (^{1}C^{2}, ^{505}C^{506}, ^{529}C^{530} and ^{689}TAA^{690}) and one nucleotide deletion (^{10}G^{10}). Three different PCR reaction were necessary to obtain the final frame shift PCR amplicon that was cloned, using *Nco*I and *Bam*HI restriction sites, into Rep5 viral vector obtaining Rep5-FSp32p13 clone.
All constructs and PCR fragments were characterized by restriction fragment analysis and sequencing.

2.2 Viral inoculations

Run-off transcripts were produced and inoculated together with the helper strain Stras12, containing BNYVV RNA-1 and -2 (Quillet et al., 1989), onto *C. quinoa* leaves, as previously described by Klein et al. (2007).
A transmission test, adapted from Koenig and Stein (1990), has been developed on *B. vulgaris* plants using aviruliferous *P. betae*. Seeds of rhizomania-susceptible sugar beet cultivars were planted on sterile sand. To produce sap inoculum, *C. quinoa* leaves were rub-inoculated using BNYVV Stras12, supplemented with BNYVV RNA-3 *in vitro* transcript and with transcripts derived from tUC47 and constructs described above. Mechanical inoculations of beet's roots were performed on ten days old sugar beet seedling using sap (inoculum) of *C. quinoa* infected leaves grounded in 50 mM KH_2PO_4 buffer containing 0.8% of macaloid. A set of 10 young sugar beet plants were placed in a glass tube with a diameter of 2 cm together with 3 ml of inoculum containing 0.09 g carborundum powder and then mixed for 1 minute. Inoculated sugar beet seedlings were planted in a sterile sand box (10x10cm) and inoculated, one week later, with aviruliferous *P. betae* zoospore suspension derived from protozoa-infected sugar beet roots floated in a water solution for 2 hours. After one week, new sugar beet seeds were sow in the same box. Two months later roots from non-inoculated sugar beet plants were collected, dried and then pulverized into new sterile sand where new sugar beet seeds were sow. Finally, three week later, roots were analysed by TEM, RT-PCR, Western blot and Northern blot analyses. All plants were grown in a greenhouse at 24 °C (day) / 18°C (night) with a photoperiod of 16h.

2.3 Analysis of infection products

Total RNA contents were isolated from analysed samples using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Encapsidated RNAs were obtained using Protocol TM (Jupin et al., 1990). BSBMV RNA-4 northern blot analyses were performed using random ^{32}P-labeled DNA probes corresponding to nucleotides 6 to 603 for 5' probe and nucleotides 926 to 1728 for 3' probe whereas BNYVV RNAs analysis was performed as previously described (Link et al., 2005); (Schmidlin et al., 2005). Virus and *P. betae* infections were detected by RT-PCR as described (Ratti et al., 2005). Viral proteins were detected by western blotting after SDS-PAGE separation of total protein extracts from symptomatic samples and whole asymptomatic leaves or roots as described by Link et al. (2005) using rabbit anti-BNYVV CP raised against the BNYVV coat protein. Rabbit Anti-Flag polyclonal IgG and mouse Anti-Flag monoclonal antibody or Anti-Flag-Peroxidase monoclonal antibody (Sigma-Aldrich, MO, USA) were also used.

2.4 Sequence analyses

Sequences were treated with Vector NTI advance 11.5 software (Invitrogen Corp., Carlsbad, CA) and/or MacVector 11.1 software (MacVector Inc., Cary, NC).

Results

3.1 BSBMV RNA-4 full length clone

An unexpected 1,730 nucleotides (nts) long BSBMV RNA-4 (GenBank ID: FJ424610) has been detected from roots of sugar beet plants grown on BSBMV infested soil. Full-length BSBMV RNA-4 cDNA clone sequence analysis revealed high identity (~100%) with the previously published BSBMV RNA-4 sequence (GenBank ID: NC_003508) between nucleotides 1-608 and 1,138-1730 with 5 nts substitutions and 2 nts deletion. One nucleotide substitutions ($T^{219}C$) and two C nucleotides deletion ($^{269}C^{269}$ and $^{297}C^{297}$) were found in the 5' UTR, one nucleotide substitution was found in the ORF1 ($C^{521}T$) leading a

silent mutation. Finally three nucleotides substitution were found in the 3' UTR ($T^{1572}C$, $T^{1575}C$ and $G^{1594}T$). However, the new BSBMV RNA-4 form presented 529 additionally nucleotides between positions 608 – 1,138 (Fig. II.1). Coremin sequence (Ratti et al., 2009) has been identified between nts 1304 and 1323 (Fig. II.3).

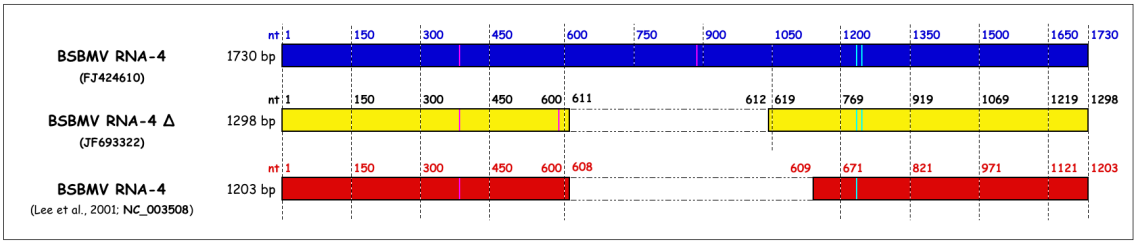

Fig. II.1 BSBMV RNA-4 sequences comparison. New BSBMV RNA-4 form (GenBank ID: FJ424610) is compared with RNA sequence described by Lee et al. (2001, GenBank ID: NC_003508) and with an RNA4 deleted form (GenBank ID: JF693322) obtained after 14 serial mechanical inoculations experiments.

In vitro transcripts produced from the full length infectious BSBMV RNA-4 cDNA clone pUC47 (tUC47) were rub-inoculated, together with BNYVV Stras12 helper strain (Quillet et al., 1989), onto *Chenopodium quinoa* plant leaves. Typical chlorotic spots appeared 7 days post inoculation (dpi) on leaves inoculated with Stras12 helper strain but local necrotic spots appeared when tUC47 transcripts were added (Fig. II.2 (a)). Effective replication of BSBMV RNA-4 has been confirmed using northern blot analysis on both total RNAs and encapsidated viral RNA using BNYVV RNA-1 and -2 and BSBMV RNA-4 specific radiolabelled probes (Fig. II.2 (b)). BSBMV RNA-4 progeny was indeed detected within different inoculated plants as *Tetragonia expansa* and *Beta macorcarpa*, indicating, as for BSBMV RNA-3 (Ratti et al., 2009), the efficient replication of BSBMV RNA-4 by BNYVV RNA-1 and -2 complex. Encapsidation of BSBMV RNA-4 by the BNYVV coat protein was confirmed by TM extraction protocol, (Jupin et al., 1990) and virus purification (Tamada and Abe, 1989).

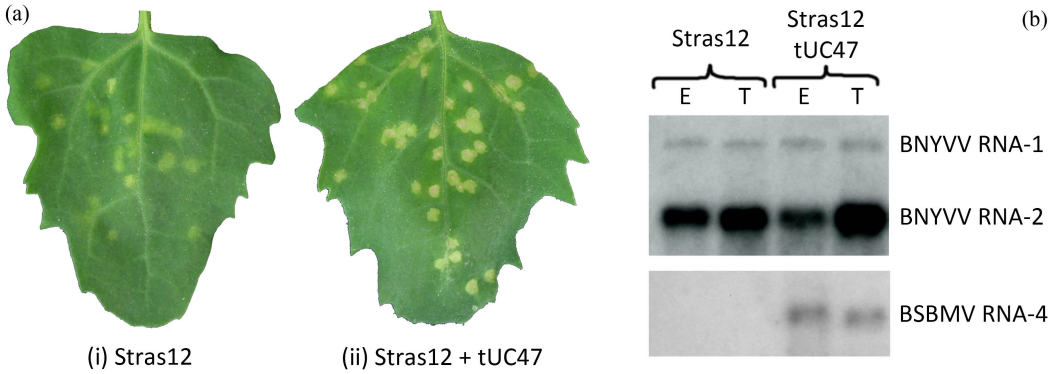

Fig. II.2 (a) Symptoms on *C. quinoa* leaves obtained 7 dpi after rub inoculation of BNYVV RNA-1 ad -2 (i) supplemented with full-length transcripts of BSBMV RNA-4 (tUC47) that induces necrosis spots (ii).
(b) Northern blot analyses of *C. quinoa* lesions contents induced (7 dpi) by Stras12 helper strain alone (lanes 1 and 2) or supplemented with transcripts of BSBMV RNA-4 (tUC47; lanes 3 and 4). Two different RNAs protocol extraction were used: (T) Trizol protocol or (E) TM protocol. RNAs were detected using riboprobes complementary to BNYVV RNA-1 and -2 (upper panel) or to 5' end of BSBMV RNA-4 (lower panel).

3.2 Deleted forms of BSBMV RNAs

Shortened forms of both BNYVV RNA-3 and RNA-4 have been reported after serial mechanical inoculation to *C. quinoa* leaves (Bouzoubaa et al., 1991). Sap of BSBMV infected sugar beet roots was used for the first mechanical transmission of a 7 days serial inoculation of the virus onto leaves of *C. quinoa* plants. At every passage, total RNA was extracted from a part of inoculated leaves from which cDNA was obtained using OLIGOdT25 Bgl II primer. The same primer was then coupled with specific forward primer designed at 5' end of BSBMV RNA-3 or BSBMV RNA-4 in a PCR reaction.

A deleted form of BSBMV RNA-4 (1,298 nts, GenBank ID: JF693322) appeared after 14 passages (Fig. 1) and its sequence analysis showed deletion of 433 nucleotides between positions 611 and 1,044 of full-length BSBMV RNA-4 (GenBank ID: FJ424610) and two nucleotides substitution in the p32 coding sequence ($C^{521}T$ and $T^{1130}G$) leading to silent and $W^{250}G$ mutations, respectively. Moreover BSBMV RNA-4 deleted form shows a single putative ORF that share N-terminal amino acid sequence (1 – 76 aa) and C-terminal amino acid sequence (221 – 283 aa) with p32 coding sequence. Interestingly, the putative ORF of BSBMV RNA-4 previously described (Lee et al., 2001) also share N-terminal amino acid sequence (1 – 75 aa) with p32 coding sequence but shows a completely different sequence (6.2% amino acids identity score) in the last 33 amino acids of C-terminal part.

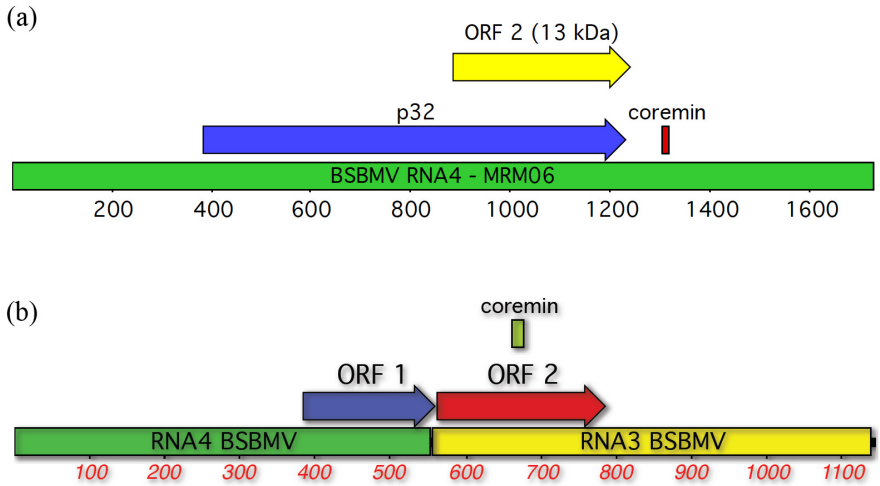

Fig. II.3 Genome organization. (a) BSBMV RNA-4. Coremin seq uence is present between nts 1304 and 1323. (b) Chimeric BSBMV RNA. ORF-1 and -2 have predicted masses of 7 and 9 kDa, respectively. Coremin sequence is present between nts 662 and 681.

A chimeric form of BSBMV RNAs -4 and -3 (1,142 nts, GenBank ID: JF513084) was also detected after 21 serial mechanical inoculated passages and remained unaltered up to fortieth passage (Fig. II.3 (b)). Sequence analysis of BSBMV Chimeric RNA revealed that

nucleotides 1 to 557 were from BSBMV RNA-4 (1 – 557 nts of FJ424610) and nucleotides 557 to 1,142 were from BSBMV RNA-3 (1,138 – 1,720 nts of EU410955). Such chimeric RNA encodes putatively two ORFs. The first has a predicted size of 7 kDa corresponding to the first 60 aa of BSBMV p32 (nucleotides 383 – 562), the second ORF (nucleotides 562 – 789) encodes a 9 kDa polypeptide corresponding to the 76 C-terminal amino acids of the additional putative ORF described on BSBMV RNA-3 (Gilmer and Ratti, in press). Nucleotides sequence comparison of BSBMV RNA-3 and Chimeric RNA evidenced just one nucleotide substitution ($A^{1156}T$), leading to $K^{25}M$ mutation, within the putative 9 kDa protein. Moreover coremin sequence is present between nts 662 and 681 (Fig. II.3).
No deleted forms of BSBMV RNA-3 have been reported during each RT-PCR analyses performed until fortieth serial mechanical inoculation passage.

3.3 BSBMV RNA-4 encoded proteins

Expression of the two putative proteins, p32 and p13, encoded by the new form of BSBMV RNA-4 were investigated *in planta*. The FLAG epitope sequence was fused in frame at the N-terminus of p32, p13 and BNYVV p31 proteins and cloned into Rep3 and Rep5 viral vectors. When inoculated onto *C. quinoa* leaves, together with Stras12 helper strain, the Rep3-Flag-p32 transcripts induced necrotic local lesions 7 dpi, identical to those induced by Stras12 + tUC47. Symptoms induced by Stras12 + Rep3-Flag-p13 transcripts did not differ from those induced in the absence of Rep3-Flag-p13. Expression of BNYVV p31 protein, using Rep3 viral vector, induces necrotic ring spot lesions comparable to those we obtained using full length BNYVV RNA-4 clone (pUC-4B) in *C. quinoa* leaves. Identical results were obtained performing the same experiments using Rep5 viral vector expressing p32, p13 and p31 proteins with or without FLAG epitope.
When Rep5-FSp32p13 transcripts were used in the presence of BNYVV Stras12 helper strain, chlorotic local lesions were produced on *C. quinoa* leaves, indicating that the p32 expression was responsible for the necrotic local lesions induced by BSBMV RNA-4 or Rep5-p32 transcripts, thus excluding influence of RNA sequence.
Lesions from *C. quinoa* plants, inoculated with Stras12 supplemented with Rep3-Flag-p32 or Rep3-Flag-p13 transcripts, were analysed by western blot as previously described (Link et al., 2005). When FLAG-specific antibody conjugated with peroxidase was used, a protein of about 32 kDa was detected within all samples with different intensities. Such band appeared with a bigger intensity when Rep3-Flag-p32 was expressed. A band of

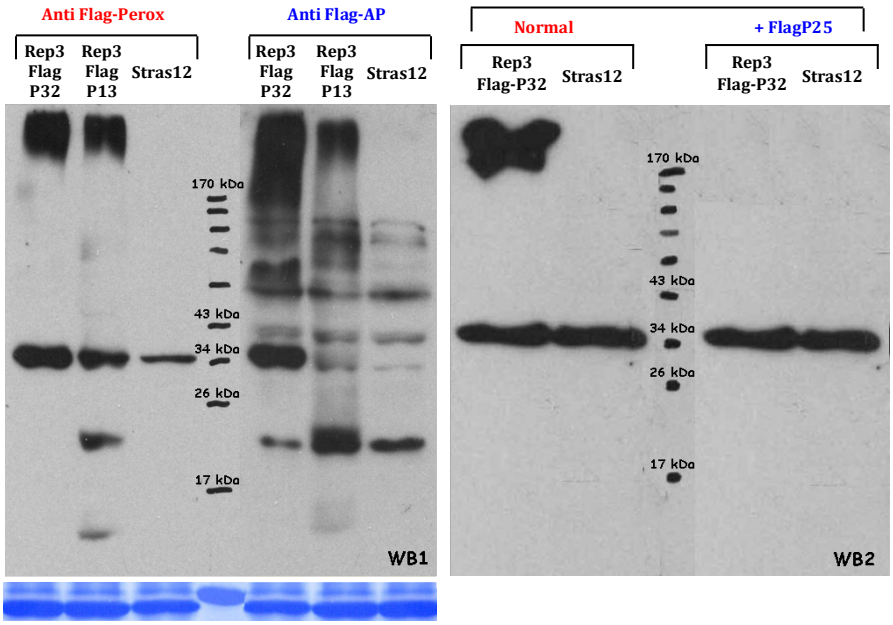

Fig. II.4 Western blot analyses of BSBMV RNA-4's ORFs expression.
WB1: *C. quinoa* lesions (7 dpi) were analyzed using anti-Flag antibody conjugated with Alkaline Phosphatase (AP) (on the left) or anti-Flag antibody conjugated with Peroxidase (on the right). Same amount of proteins extract were loaded on gels.
WB2: normal conditions (on the left) were compared with addition of specific Flag competitor (FlagP25 protein) (on the right) during membrane incubation with anti-Flag conjugate with Peroxidase.

about 13 kDa was detected in sample infected by Stras12 + tRep3-Flag-p13 (Fig. II.4 WB1, left panel). Uncharacterized yet high molecular weight products were also detected from extracts containing viral vectors expressing Flag32 and Flag13 proteins (Fig. II.4). However the use of BNYVV Flag-p25 competitor confirmed flag antibody specificity for the high molecular weight products and not for the 32 kDa protein (Fig. II.4 WB2).

A different chemistry was therefore used to detect flag tagged protein, using FLAG-specific antibodies conjugated with Alkaline Phosphatase. Such analysis permitted to conclude the exclusive expression of a 32 kDa protein only in sample containing Rep3-Flag-p32 (Fig. II.4 WB1, right panel).

Chlorotic lesions appeared on *C. quinoa* leaves inoculated with Stras12 supplemented with BSBMV RNA-4-FlagIN transcripts. Western blot analysis, performed using FLAG-specific antibody conjugated with Alkaline Phosphatase, specifically revealed a protein of about 32 kDa (Fig. II.5). Similarly, western blot analysis of necrotic ring spot lesions induced by Stras12 + tRep3-Flag-p31, specifically detected the 31 kDa protein. In this case, a high molecular weight complex previously observed in Flag-p32-infected *samples* was also detected on Flag-p31 inoculated sample (Fig. II.5).

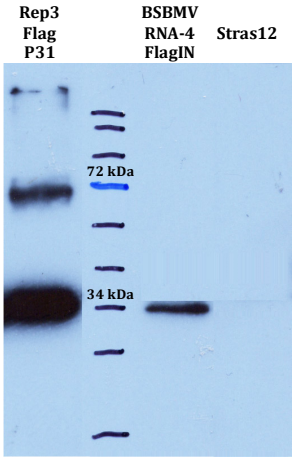

Fig. II.5 Western blot analysis using anti Flag conjugate with Alkaline Phosphatase on *C. quinoa* leaves inoculated with Stras12 + tRep3-Flag-p31 or tBSBMV RNA-4-FlagIN.

3.4 BSBMV RNA-4 doesn't affect long-distance movement of the virus in B. macrocarpa

Ten days-old *B. macrocarpa* seedlings were inoculated with Stras12 helper strain supplemented with t35 (BNYVV RNA-3) or tUC31 (BSBMV RNA-3) and tUC31 plus tUC47. Plants were also inoculated with total RNA extracted from BSBMV-infected sugar beet roots that were tested free from other beet soil-borne viruses by RT-PCR multiplex analysis (Meunier et al., 2003). As reported in Table II.2 yellow mosaic symptoms began to appear 10-14 dpi on systemic leaves of plants inoculated with Stras12 supplemented with BNYVV RNA-3 or with wild type BSBMV isolate. No difference were observed on time necessary for systemic symptoms appearance (15-22 dpi) when Stras12 was supplemented with BSBMV RNA-3 or BSBMV RNA-3 plus -4, whereas no symptoms nor viral RNA was detected 30 dpi in the Stras12 inoculated plants. Therefore, the

Inoculum	Symptoms (dpi)		Efficiency of systemic movement
	Local	Systemic	
Stras12	5-7	-	0
Stras12 + t35	5-7	10-14	>90
Stras12 + tUC31	5-7	14-21	>70
Stras12 + tUC31 + tUC47	5-7	14-21	>70
Stras12 + tUC47	5-7	-	0
BSBMV isolate	5-7	10-14	>90

Table II.2 The Efficiency of viral systemic spread. Kinetics of *B. macrocarpa* local and systemic symptoms appearance after the inoculation of BNYVV Stras12 helper strain supplemented with BNYVV RNA-3 transcripts (t35), BSBMV RNA-3 transcripts (tUC31) alone or with BSBMV RNA-4 transcripts (tUC47) compared to wild type BSBMV infection. The efficiency of viral systemic spreading corresponds to the average of systemically infected plants versus total inoculated plants. 10 different *B. macrocarpa* plants were inoculated for each inoculum.
dpi day post inoculation; - no symptoms detection

combination of two coremin sequences, supplied by BSBMV RNA-3 and -4, did not increased a systemic movement's efficiency.

3.5 BSBMV RNA-4 p32 protein supports virus transmission through P. betae

Considering high correlation between BNYVV and BSBMV viruses, demonstrated by capability of BNYVV viral machinery to replicate and encapsidate BSBMV RNA-3 (Ratti et al., 2009) and BSBMV RNA-4 (this study), the possible complementation between BSBMV and BNYVV RNAs-4 has been investigated for an efficient transmission of BNYVV RNAs through the vector *P. betae* in *B. vulgaris* plants.

Transmission experiments were performed using Stras12 isolate + t35 (called Stras123), as negative control, and Stras123 in combinations with three different transcripts obtained from pUC47, deleted BSBMV RNA-4 (pUB47 Δ) and full length BNYVV RNA-4 (pUC-4B), as positive control (Table II.3).

	***C. quinoa* symptoms**	**TEM**	**mRT-PCR**		**WB**
			P. betae	**RNA-2**	
Stras12	CS	-	+	-	-
Stras123 + tUC-4B	NR	+	+	+	+
Stras12 + tUC47	NS	+	+	+	+
Stras123 + tUC47	NS	+	+	+	+
Stras12 + tUC47 Δ	CS	-	+	-	-
Stras123 + tUC47 Δ	CS	-	+	-	-
Stras123	YS	-	+	-	-
Stras123 + tRep5-FSp32p13	YS	-	+	-	-
Stras123 + tRep5-p32	NS	+	+	+	+
Stras123 + tRep5-p31	NR	+	+	+	+

Table II.3 Analysis of virus transmission through the vector *P. betae* performed with different viral infection combination from inoculated *C. quinoa* leaves. Test analyses were performed with Transmission Electron Microscopy (TEM), multiplex RT-PCR and Western blot using anti BNYVV CP antibody.
CS = chlorotic spots; YS = yellow spots; NS = necrotic spots; NR = necrotic ring spots.

Stras123 with full length BSBMV or BNYVV RNAs-4 were successfully transmitted by *P. betae* to roots of healthy sugar beet plants on which rod-shaped viral particles were identified by Transmission Electron Microscopy (TEM) analysis, BNYVV coat protein expression was demonstrated by western blot and BNYVV or BSBMV RNAs replication was confirmed by northern Blotting. No virus transmission occurred using Stras123 or Stras12 + deleted form of BSBMV RNA-4.

Previous studies revealed competition between BNYVV and BSBMV RNAs-3 as well as between RNAs-3-derived replicons (Lauber et al., 1999); (Ratti et al., 2009). Therefore, Rep5 viral vector has been employed to express p32 or p31 proteins during transmission tests. Transcripts from Rep5-p32, Rep5-p31, Rep5-FSp32 and from empty Rep5 viral vector were used, together with Stras123 (Table II.3), in order to investigate the essential role of both proteins, out of their RNA context, in the efficient viral transmission. TEM, western blot and RT-PCR analyses confirmed that both p32 and p31 proteins could support *P. betae* transmission when expressed by Rep5 vector. Neither coat protein nor viral particles were detected or observed in the sample inoculated with p32 frame shift protein (Fig. II.6).

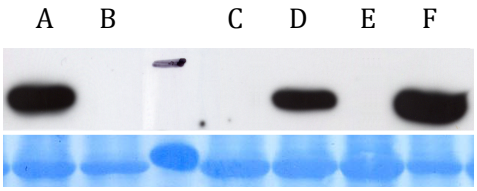

Fig. II.6 Western blot analysis using anti BNYVV CP antibody on sugar beet roots samples from transmission test. (A) Stras123 + tUC47; (B) Stras123 + tUC47 Δ; (C) Stras123; (D) Stras123 + tRep5-p32; (E) Stras123 + tRep5-FSp32p13; (F) Stras123 + tRep5-p31.

Discussion

4.1 BSBMV RNA-4 is 1,730 nts long and it's replicated by BNYVV machinery

Internal deletions or loss of smaller RNA species have been described for BNYVV isolates maintained by repeated mechanical inoculation of local lesion hosts (Hehn et al., 1994). Prolonged cultivation of field-infected plants especially when growth at high temperatures results also in the spontaneous deletion of part of *Soil-borne wheat mosaic virus* RNA2 (Jianping et al., 1995). Our study demonstrated that BSBMV RNA-4 is longer than the form previously isolated from leaves of field-infected sugar beet plants maintained in the greenhouse for indefinite period. The new BSBMV RNA-4 form shares 47 % nucleotide sequence identity with BNYVV RNA-4 instead of 55 % identity of BSBMV RNA-4 described by Lee et al., (2001). Furthermore, shorter RNA-4 specie highly similar to the sequence described in 2001, appeared after several serial mechanical inoculations (Fig. 1). According to sequence analysis we can speculate on the hypothesis that a spontaneous deletion mutant, similar to the BSBMV RNA-4 deleted form obtained during this study, was previously described by Lee et al., (2001).

Moreover 3′ UTR sequence of the new full length BSBMV RNA-4, as well as all the other BSBMV RNAs, resulted highly conserved and comparable to the sequence of BNYVV RNAs. As previously reported viral RNA selection and replication mechanism appear to be conserved for both viruses. It has been demonstrated that compensatory mutations within 3′ UTR sequences of BSBMV RNA-3 validate the secondary structure essential for RNA replication making BNYVV RNAs-1 and -2 capable to replicate BSBMV RNA-3 (Ratti et al., 2009). Detection of BSBMV RNA-4 progeny, after the inoculation of BSBMV RNA-4 transcripts together with BNYVV helper strain, indicated that BNYVV RdRp can indeed replicate BSBMV RNA-4. Moreover, BSBMV RNA-4 was also encapsidated by BNYVV capsid proteins, as it was protected during the TM extraction and virus purification processes.

4.2 Recombination occur after several serial mechanical inoculations between BSBMV RNAs

RNA recombination is a process that joins non-contiguous RNA segments together. Viral RNA recombination is thought to occur when the viral replicase accidentally switches templates during complementary RNA synthesis (Lai, 1992); (Nagy and Pogany, 2000).
The resulting novel combinations of genes, sequence motifs, and/or regulatory RNA sequences could cause dramatic changes in the infectious properties of RNA viruses that can potentially lead to the emergence of new viruses or strains. Therefore, RNA recombination can help viruses to escape natural resistance mechanisms contributing to viral outbreaks (Cheng et al., 2006).
As described above appearance of BNYVV RNA-3 and -4 deleted forms has been reported after successive mechanical inoculation to *C. quinoa* leaves (Bouzoubaa et al., 1991) but, to our knoledge, no natural recombinant RNAs (Chimeras) have been described on viral species belonging to genus *Benyvirus*. In this paper we therefore report for the first time a natural Chimeric RNA derived from recombination process between BSBMV RNAs -4 and -3 that occurred during serial mechanical inoculation on *C. quinoa* leaves. Study of the chimeric RNA influence on BSBMV fitness was not part of the present study otherwise sequence analysis revealed two putative ORFs encoding small proteins which could be important for BSBMV adaptability. Interestingly, chimeric RNA escapes, during replication process, from competition with BNYVV or BSBMV RNA-3 (data not shown). This characteristic makes chimeric RNA the best candidate to become the basis of a new viral vector able to ensure co-expression of 3 different foreign proteins when used together with previous described Rep3 or RepIII and Rep5 replicons (Bleykasten-Grosshans et al., 1997); (Schmidlin et al., 2005); (Ratti et al., 2009).

4.3 BSBMV RNA-4 p32 encoded protein enhances symptoms expression

BNYVV RNA-4 produced strong chlorotic lesions in *Tetragonia expansa* leaves and leaf stunting, curling and severe mosaic with leaf distortions on *Nicotiana benthamiana*. BNYVV p31 plays a multifunctional role in efficient vector transmission, enhanced symptom expression and root-specific silencing suppression (Rahim et al., 2007). BSBMV

RNA-4 encoded p32 share relevant amino acid identity (49.8 %) with B-type BNYVV p31 that increase to 68.4 % in terms of amino acid similarity suggesting a similar role of BSBMV RNA-4 on symptoms expression.

In our experiments full length BSBMV RNA-4, as well as p32 protein expressed by Rep3, have been associated, on *C. quinoa* leaves, to induction of necrotic spots while necrotic ring spots are been observed when BNYVV p31 was expressed by Rep3.

It is known that viral proteins associated with symptom severity often show silencing suppression activity, as for BNYVV p31, (Rahim et al., 2007) a possible activity of p32 as a suppressor of RNA silencing cannot be excluded. A rapid screening of p32 protein performed according to the protocol developed by Guilley et al. (2009) evidence no RNA silencing suppressor activity in leaf (Data not shown) but deep studies are necessary to evaluate p32 activity in root context..

Western blot analysis identified a specific high molecular weight products on sample inoculated with FlagP32 suggesting p32 interaction with other viral or plants protein(s) may mediated by post translational modifications. Additionally, when presence of Flag sequence in the middle of the p32 ORF (BSBMV RNA-4-FlagIN clone) probably modify p32 structure preventing its activity, different symptoms (chlorotic spots) has been observed on *C. quinoa* leaves and high molecular weight products are not detected on Western blot analysis.

4.4 BSBMV RNA-4 is not involved on long distance movement

In *B. macrocarpa*, BNYVV long distance movement is related to RNA-3 presence and involves a nucleotide sequence named “core” (Lauber et al., 1998). A conserved “coremin” sequence has been identified within core motif of BNYVV and BSBMV RNAs-3, in BNYVV RNA-5 and BSBMV RNA-4 as well as in other viral species belonging to the genus *Cucumovirus*. BSBMV RNA-3 was recently reported as less efficient element for long-distance movement of BNYVV RNA-1 and -2 in *B. macrocarpa* plants when compared to wild type BSBMV isolate (Ratti et al., 2009) then the presence of two motifs within BSBMV (RNA-3 and -4) was indicated as one possible explanation for this observation. As demonstrated by the results obtained during our experiments, the presence of a second coremin sequence is not able to influence, positively or negatively, the long-distance movement efficiency. Moreover, even if BSBMV RNA-4 contains a coremin sequence, such

RNA species cannot replace RNA-3 for long distance movement on *B. macrocarpa* (Table 2). A possible implication of other genomic RNA structural motifs present in *cis* (Miller and White, 2006) or in *trans* on BSBMV RNA-1 and/or RNA-2 may favour long distance movement.

4.5 BSBMV RNA-4 encoded p32 is essential for vector transmission

Knowledge regarding benyviruses transmission through the vector *P. betae* has been up to now based on advances obtained by BNYVV studies. Previous study showed that wild-type (wt) BNYVV RNA-4 is required for efficient transmission of BNYVV by the plasmodiophorid *P. betae* (Tamada and Abe, 1989), moreover BNYVV RNA-4 deleted forms encoding truncated p31 protein are not able to transmit the virus (Rahim et al., 2007). No information was available about the role of BSBMV RNA-4. In this study we demonstrated that full length BSBMV RNA-4 is indeed essential for virus transmission through the vector. We also evidenced a possible complementation between BNYVV RNA-4 and BSBMV RNA-4 for BNYVV RNAs -1, -2 and -3 acquisition and efficient transmission through *P. betae* in *Beta vulgaris* plants.

No data are available about ability of BSBMV RNA-4 described by (Lee et al., 2001) to mediate virus transmission through the vector. However, in our experiments, we demonstrated that in the presence of a deleted BSBMV RNA-4, obtained after several serial mechanical inoculation passages and highly similar to previous described BSBMV RNA-4, *P. betae* cannot transmits BNYVV RNAs.

Finally, through expression of the BSBMV p32 and BNYVV p31 proteins with Rep5 viral vector, we demonstrated for the first time that the BSBMV p32 or BNYVV p31 proteins, and not RNAs-4, are definitively the transmission determinant for Benyviruses.

More experiments will be then needed to better investigate the role of BSBMV p32 protein and understand the molecular mechanism occurring between Benyviruses and vector, in particular discovery of domain(s) involved on virus transmission could address new strategies to develop breeding programs for rhizomania resistance.

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Chapter 3

BSBMV p32 protein analysis

Introduction

As described on Chapter 1, viruses developed different strategies to enable and regulate efficiently the expression of their own proteins from the compacted molecules composing their genomes. Minimizing the size of functional genome often requires multifunctional proteins with activities regulated potentially with post-translation modifications (PTMs).

During this thesis we identify a new full-length form of BSBMV RNA-4. On Chapter 2, we demonstrated that it is amplified *in planta* by BNYVV viral machinery and that it can substitute BNYVV RNA-4 for an efficient transmission through the vector *P. betae* in *B. vulgaris* plants. Two putative ORFs have been found on RNA-4 sequence that could encode for a 32 kDa (p32) and a 13 kDa (p13) products. We showed that the expression of BSBMV p32 protein by itself is essential and sufficient for the viral transmission.
We started a molecular and functional characterization of BSBMV p32 protein to better investigate its role during viral transmission.

Material and methods

2.1 Clones production

Full-length cDNA infectious clone of BSBMV RNA-4 (pUC47), obtained in this thesis and described on Chapter 2, constituted the base from which many clones have been produced to investigate p32 and p13 proteins expressions using different proteins tags.
HA epitope tag (YPYDVPDYA) was fused at N-terminal, C-terminal, both extremities or as double HA tag at the N-terminus of p32 and p13 proteins. Flag epitope tag (DYKDDDDK) was also used similarly. Different PCR reactions were performed using specific primer pairs (see Appendix B) carrying *Nco*I or *Bam*HI restriction sites added on forward or reverse primers, respectively, to facilitate cloning procedures into Rep3 viral vector (Bleykasten-Grosshans et al., 1997) and produce the clones listed in Table III.1.

Tag	Zone	Protein	Clone
HA	N-term	P32 or P13	MD1 or MD5
HA	C-term	P32 or P13	MD2 or MD6
HA	N-term and C-term	P32 or P13	MD3 or MD7
2xHA $^{\Delta}$	N-term	P32 or P13	MD4 or MD8
Flag	N-term	P32 or P13	MDS1 or MDS6

Table III.1 Rep3 clones with P32 or P13 tagged with HA or Flag.
$^{\Delta}$ Gly and Pro amino acids were insert between two HA nucleotide sequence.

P32's deleted forms have been produced as shown in Fig. III.1. Appropriate primer pairs (Appendix B) have been used to obtain PCR products that were digested by *Nco*I and *Bam*HI and then cloned inside Rep3 viral vector obtaining six different clones (p32Δ1 to Δ6). Two series of clones were produced, one serie with FLAG at N-terminal and one other with HA at C-terminal.

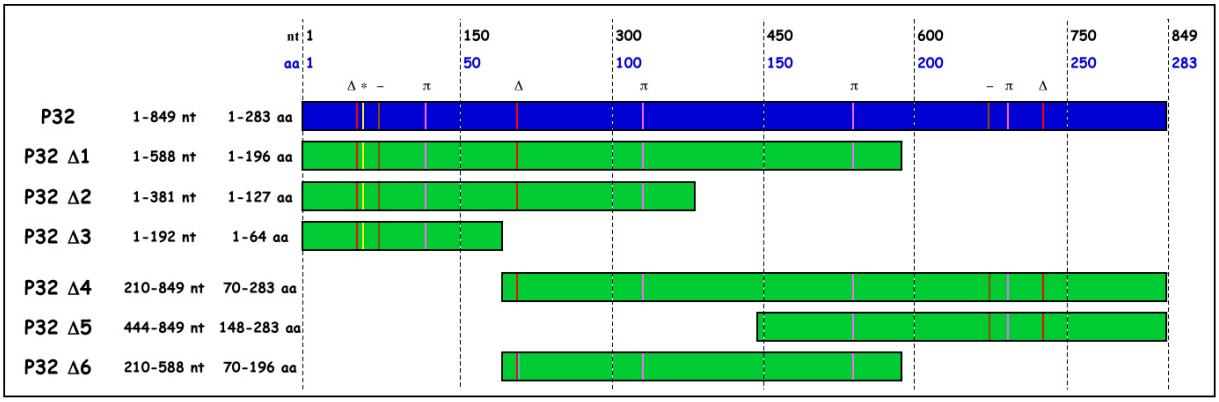

Fig. III.1 P32's deleted forms. Blue bar represents wild type P32; Green bars represent deleted forms. Prosite motif are shown: Δ N-glycosylation site (red line); * Casein kinase II phosphorylation site (yellow line); - Protein kinase C phosphorylation (brown line); π Myristoylation site (purple line).

BNYVV Rep3-GFP and Rep5-GFP were obtained from Schmidlin et al. (2005). Fusion between GFP and p32 protein were generated by overlap-extension mutagenesis (Ho et al., 1989) of cloned cDNA by PCR. In Rep3-GFP-P32 and Rep3-GFP-P13 clones, the GFP termination codon was replaced by CCC and the first P32 or P13 initiation codon by GGG; in Rep3-P32-GFP and Rep3-P13-GFP the P32 or P13 termination codon was replace by CCC and the GFP initiation codon by GGG.

PCR site-directed mutagenesis using specific primers (Appendix B) were performed to mutate predicted post translational modification sites ^{18}NVTG^{21} , ^{69}NVSV^{72} and ^{39}GVLCNI^{44} on the P32 protein sequence. PCR products were digested using *Nco*I and *Bam*HI restriction enzymes and cloned into *Nco*I-*Bam*HI-digested Rep3 viral vector obtaining clone reported in Table III.2.

Clone	Construction	Mutation
MD150	Rep3-p32 N18G	$N^{18}G \Rightarrow M1$
MD151	Rep3-p32 N68G	$N^{69}G \Rightarrow M2$
MD157	Rep3-p32 N18G N69G	$N^{18}G$, $N^{69}G \Rightarrow M1, M2$
MD168	Rep3-p32 G37V-G39V	$G^{37}V$, $G^{39}V \Rightarrow M3$
MD169	Rep3-p32 N18G-G37V-G39V	$N^{18}G$, $G^{37}V$, $G^{39}V \Rightarrow M1, M3$

Table III.2 P32 mutated sequences expressed in Rep3

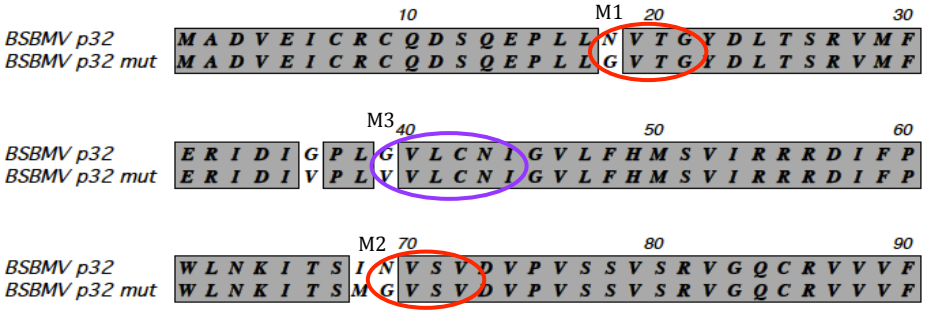

Fig. III.2 Sequence alignment of wild type and mutated BSBMV p32. Red circle and purple circle are indicated the sequence predicted site for N-glycosylated and myristoylation site, respectively.

P32, GFP-p32 and p32-GFP were obtained from Rep5-p32, Rep4-GFP-p32 and Rep3-p32-GFP clones with *Nco*I and *Bam*HI restriction enzymes and subcloned into *Sma*I-digested pBIN61 vector plasmid after Klenow fragment treatment and thus located between constitutive *Cauliflower mosaic virus* (CaMV) 35S RNA promoter and nopaline synthase transcription termination sequence (Nos).

All constructs and PCR fragments were characterized by restriction fragment analysis and sequencing.

2.2 P32 protein expression and analysis

FlagP32 and P32 proteins were extracted form Rep3-FlagP32 and Rep5-P32 plasmids, respectively, and then subcloned into pET-3d expression vector (Novagen) using *Nco*I and *Bam*HI restriction sites. Specific primer with *Bam*HI and *Eco*RI sites respectively at 5'- and 3' end (Appendix B) were used to amplify P32 nucleotide sequence, digested PCR products was then cloned into pGEX2tk expression vector (GE Healthcare) on which inserted protein was fused at C-terminal of glutathione-S-transferase (GST). To obtain the GST-p32 fusion product, the first ATG codon on p32 nucleotide sequence was replaced with GGG codon encoding Proline amino acid. Induction of recombinant protein expression was performed using different *E. coli* strains (C41, C43, BL21(DE3)pLysS).

Expression of BNYVV p42 from clone pET42 (Bleykasten et al., 1996) was used as positive control. Single *E. coli* colonies containing appropriate plasmids were grown O/N at 30 °C in 5 mL of LB medium (Sambrook et al., 1989), containing 50 µg/ml ampicillin and 25 µg/ml chloramphenicol. 100 µl of the culture were used to inoculate 10 ml of fresh LB medium then grown to OD_{600} = 0.5 – 0.6. Protein expression was induced with IPTG (Isopropil β-D-1-tiogalattopiranoside) at concentration of 0.1 mM, 0.5 mM and 1 mM and samples were collected after 1, 2 and 4 hours.

E. coli expression of p32 or p29 fused at N-terminal with Halo tag (Promega) was also performed. Amplicons of p32 and p29 were obtained by PCR form pUC47 (BSBMV RNA-4) and pUC31 (BSBMV RNA-3) clones using appropriate primer pairs (Table III.3) and then cloned into pFN18K expression plasmid vector (Promega) between *Sgf*I and *Pme*I restriction enzyme sites to obtain MD214 and MD215 clones, respectively.

In vivo expression of MD214 and MD215, were performed using *E. coli* KRX strain (Promega) according to manufacturer's protocol.

For Western blot analyses bacterial pellet was washed twice in ice-cold washing buffer (10 mM Tris-HCl at pH 7.5 and 10 mM EDTA) and bacteria were then lysed in 1 x Laemmli buffer boiling it for 3 minutes (Laemmli, 1970). Protein pattern were analysed by SDS-PAGE using coomasie blue stain or Western blot analysis using Anti-Flag-Peroxidase monoclonal antibody (Sigma-Aldrich, MO, USA) or Anti-Halo antibody (Promega) (Appendix A).

Primer	Sequence	PCR amplicon
BSBMV p32 SgfI F	ACC*GCGATCGC*CATGGCCGATGTGGAGATTTG	874 bp
BSBMV p32 PmeI R	ATG*GTTTAAAC*TCATGAAAATCTTGTTCGAAACAAAAC	
BSBMV p29 SgfI F	ACC*GCGATCGC*CATGGATTTGAATACTATGATG	796 bp
BSBMV p29 PmeI R	ATG*GTTTAAAC*TCACCATCATCTGTGTTAG	

Table III.3 Primers used for P32 and P29 cloning into pFN18K vector plasmid for Halo N-terminal protein expression. In red and italic are reported restriction enzymes. In blue are stop codons.

2.3 Yeast Two-hybrid systems

Full length and deleted forms of p32 sequence described in fig. III.1 were tested for transcription activation and self-interaction by using the Matchmaker yeast two-hybrid system (BD Biosciences Clontech) as previously described (Link et al., 2005). We used

pGAD424 and pGBT9 (Clontech) plasmids that carry, respectively, Activation Domain and Binding Domain of Gal4 transcription factor. Appropriate primers pairs (Table III.4), carrying *Eco*RI and *Sal*I restriction sites, were used to amplify and then subclone full length and deleted forms of p32 on pGAD424 and pGBT9 plasmids. Yeast strain HFT7c and Y187 were transformed with pGBT9- and pGAD-derived clones, respectively.

Primer	Sequence	Restriction enzyme
BSBMV p32 EcoRI F	AAA*GAATTC*GCCGATGTGGAGATTTGCCG	*Eco*RI
BSBMV p32to7 SalI PstI BamHI R	AAA*GTCGAC**CTGCAG**GGATCC*TCACTTGTT AAGCCAAGGAAAAATATCACG	*Sal*I
BSBMV p32to7 SalI PstI BamHI R	AAA*GTCGAC**CTGCAG**GGATCC*TCACACGT CAACAAGTTCATCGTGGACAAC	*Sal*I
BSBMV p32to7 SalI PstI BamHI R	AAA*GTCGAC**CTGCAG**GGATCC*TCAATAGC GCCGGGGGTCACTATAAAAG	*Sal*I

Table III.4 Primers and restriction sites used to clone full length and deleted forms of p32 sequence into pGAD424 and pGBT9 plasmids. In colours and italic are restriction enzymes.

2.4 In vitro and in vivo expression

TNT Coupled Reticulocyte Lysate Systems (Promega) was used for *in-vitro* expression following manufacturer's protocol as reported in Appendix A.

In vivo expression of MD214 and MD215 clones, were performed using *E. coli* KRX strain (Promega) with some modification to the manufacturer's protocol (Single Step (KRX) Competent Cells Technical Manual - Appendix A).

Results

3.1 Bio-informatics analysis

Bioinformatics Gonnet matrix analysis (Gonnet et al., 1992) was performed to align protein sequences and to calculate identity and similarity between nucleotides or amino acids sequences of proteins encoded by BNYVV and BSBMV RNAs-4. Alignment analysis between BSBMV p32 and BNYVV p31 proteins showed amino acids identity of 49.8% and similarity of 68.4% demonstrating that p32 is much closer to p31 than to BSBMV p13 described by Lee et al. (2001) (Table III.5).

BNYVV - A type	**nt identity – RNA-4**	**aa identity – p31**	**aa similarity – p31**
BSBMV - MRM06 (RNA-4 p32)	46.1%	49.8%	68.4%
BSBMV - EA (RNA-4 p13)	54.8%	17.0%	25.9%

BSBMV – p32 protein	**N-term (1-75 aa)**		**C-term (76-283 aa)**	
	aa identity	**aa similarity**	**aa identity**	**aa similarity**
BSBMV - EA (RNA-4 p13)	100%	100%	6.2%	10.6%
BNYVV – A type (RNA-4 p31)	57.3%	76%	47.1%	64.3%
BNYVV – B type (RNA-4 p31)	56.0%	76%	47.6%	65.2%
BNYVV – P type (RNA-4 p31)	57.3%	76%	46.7%	64.8%

Table III.5 Amino acid sequence identity and similarity between BSBMV p32 and BSBMV-EA p13 and between BSBMV p32 and p31 proteins from BNYVV type A, B and P.

Amino acid sequence alignment of BSBMV p32 with p13 protein of BSBMV EA isolate and with p31 protein from BNYVV type A, B and P, reveals high identity between N-terminal regions (100% and 56-57.3%, respectively) and low identity between C-terminal regions (6.2% and 46.7-47.6 %, respectively) (Table III.5; Fig. III.3).

We investigated the presence of possible post-translation modification sites on p32 or p31 protein sequence using Prosite motif search (http://expasy.org/prosite) including patterns with high probability of occurrence. The results of the bioinformatics analysis is presented in table III.6.

Motif	Pattern	Amino acids	BSBMV p32	BNYVV p31
N-glycosylation	N [^P] [ST] [^P]	18 – 21	^{18}NVTG^{21}	–
		69 – 72	^{69}NVSV^{72}	–
		130 – 133 A	–	^{130}NITN^{133}
		133 – 136 A, B	–	^{133}NTSN^{136}
		143 – 146 A, B	–	^{143}NGSY^{146}
		225 – 228	–	^{225}NETK^{228}
		242 – 245	^{242}NRTI^{245}	–
		260 – 263	–	^{260}NTTY^{263}
N-myristoylation	G [^EDRKHPFYW] • {2} [STAGCN] [^P]	39 – 44	^{39}GVLCNI^{44}	^{39}GVLCNI^{44}
		79 – 84	–	^{79}GVGVGR^{84}
		81 – 86 B	–	^{81}GVGRAR^{86}
		98 – 103	–	^{98}GIFHGW^{103}
		106 – 111	^{106}GCFIGA^{111}	–
		108 – 113	–	^{108}GCFLNA^{113}
		180 – 185	^{180}GCAYCS^{185}	–
		182 – 187	–	^{182}GCVCCS^{187}
		223 – 228	–	^{223}GTNETK^{228}
		231 – 236	^{231}GLVGAD^{236}	–
Casein kinase II phosphorylation site	[ST] •{2} [DE]	20 – 23	^{20}TGYD^{23}	–
		92 – 95	–	^{92}TSRE^{95}
		117 – 120	–	^{117}SGVD^{120}
		178 – 181	–	^{178}SNID^{181}
		262 – 265	–	^{262}TYGD^{265}
Protein kinase C phosphorylation site	[ST] • [RK]	25 – 27	^{25}TSR^{27}	^{25}TAR^{27}
		92 – 94	–	^{92}TSR^{94}
		145 – 147	–	^{145}SYR^{147}
		225 – 227	^{225}TQR^{227}	
cAMP- and cGMP-dependent protein kinase phosphorylation site	[RK]{2} • [ST]	201 – 204	–	^{201}RRDS^{204}
Tyrosine kinase phosphorylation site	[RK] •{2,3} [DE] •{2,3} Y	54 – 61 A, P	–	^{54}RHIDVYPY^{61}
Amidation site	• G [RK] [RK]	199 – 202	–	^{199}CGRR^{202}

Table III.6 Summary of the post translation modification sites identified within the amino acid sequence of BSBMV p32 protein (ACJ60504) and BNYVV p31 type A, B and P proteins (BAA12347, AAA42800 and ABG66422).
A, B or P when present indicate that predicted modification site are present on BNYVV p31 type A, B or P.

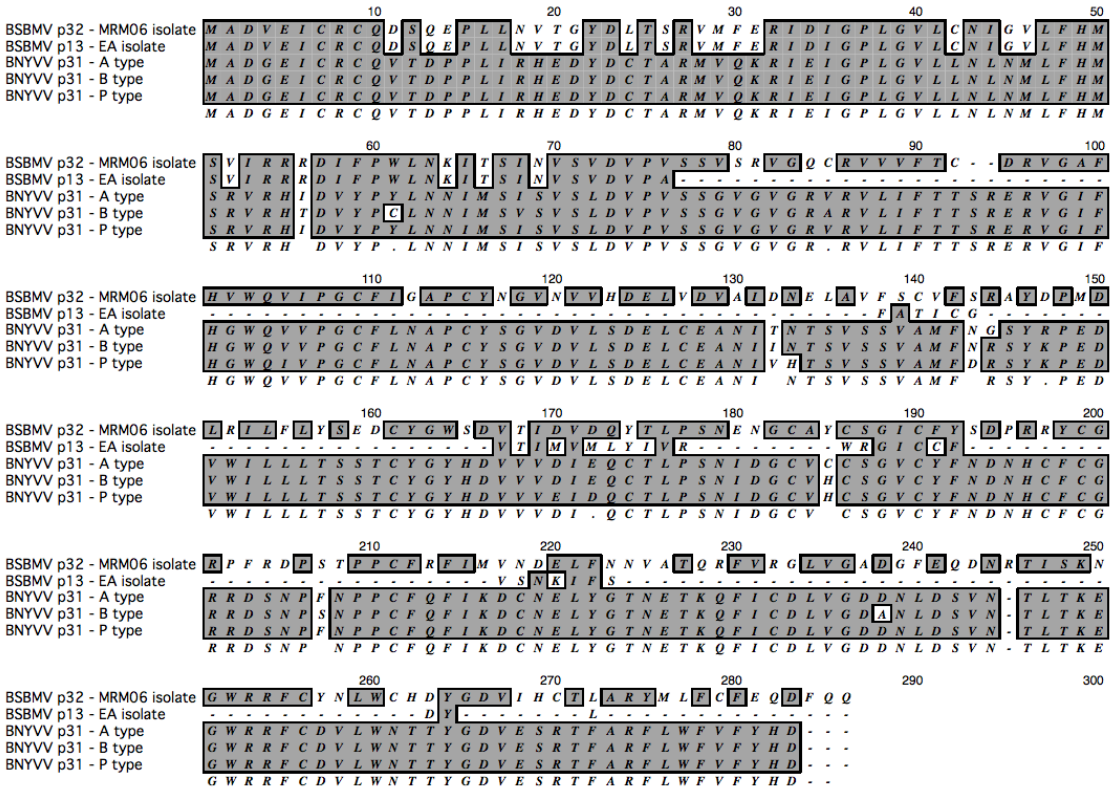

Fig. III.3 Amino acids sequence alignment between BSBMV and BNYVV RNAs-4 ORFs.

3.2 Investigating p32 protein expression using different Tags

Rep3 derived clones were linearized using *Hind*III restriction enzyme and then *in vitro* transcribed using T7 RiboMax system (Promega). Each transcript was inoculated together with the helper strain Stras12, BNYVV RNA-1 and -2 (Quillet et al., 1989) onto *C. quinoa* leaves. Symptoms were observed 7 dpi and in all cases, p32 proteins tagged with HA or Flag induced necrotic spots on *C. quinoa* leaves. Transcripts derived from Rep5-FSp32p13 clone and from all clones containing p13 protein tagged with HA or Flag induced chlorotic spots similar to those induced by Stras12 isolate alone (Fig. III.4).

Western blot analysis didn't reveal any signal using HA tag for p32 or p13 proteins (data not shown) but, as previous reported in Chapter 2, Western blot analysis, performed using anti Flag antibody conjugated with alkaline phosphatase, allowed the specific detection of a 32 kDa protein in sample infected by Stras12 + tRep3-Flag-p32. A band of about 13 kDa was as well detected in sample infected by Stras12 + tRep3-Flag-p13. Moreover a uncharacterized yet high molecular weight products was specifically detected only from extracts containing viral vectors expressing Flag-P32 and Flag-P13 proteins (Fig. II.4).

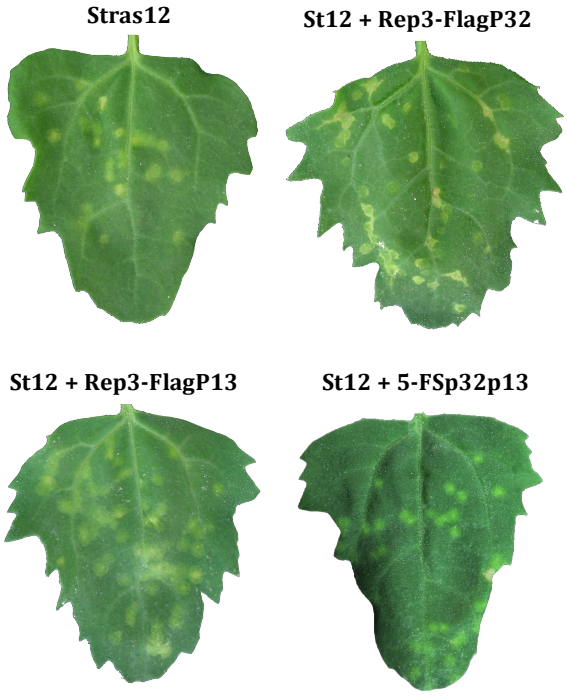

Fig. III.4 Symptoms on *C. quinoa* leaves. Necrotic spots appeared 7 d.p.i. on leaves inoculated with Stras12 plus tRep3-FlagP32.

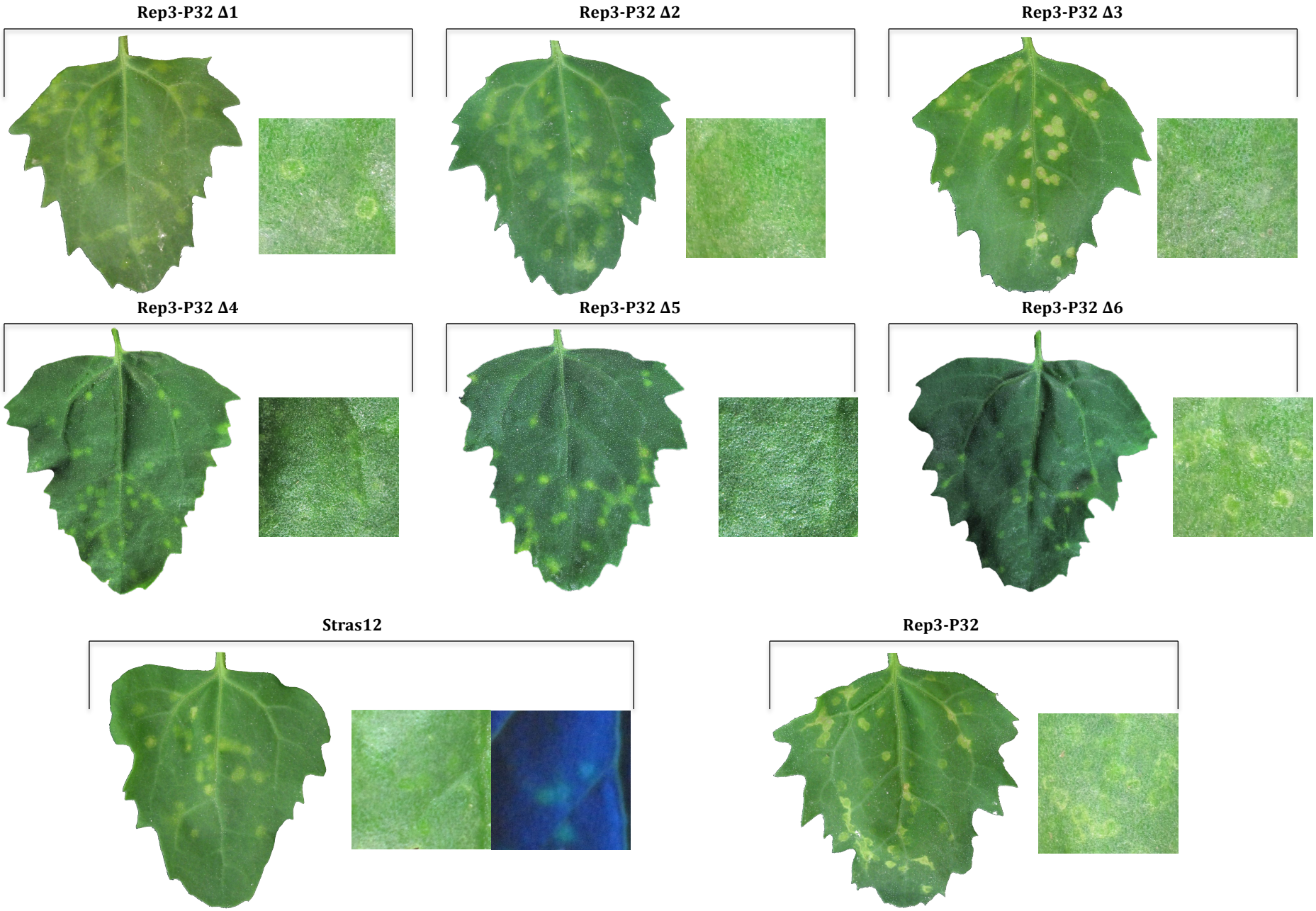

Fig. III.5 Symptoms (7 d.p.i.) on leaves of *C. quinoa* (left) and *T. expansa* (right, square) inoculated with Stras12 (BNYVV RNA-1 and -2) + tRep3-P32 Δ1-Δ6 or Rep3-P32. No different were observed when P32 or deleted protein were tagged at N-terminal with Flag or C-terminal with HA. On *T. expansa* virus spots induce by Stras12 can be observed under UV light.

3.3 P32 deleted and mutated forms

To further investigate the high molecular weight products, deleted forms of p32 were prepared. Transcripts reported on fig. III.5 were mechanically rub-inoculated, with Stras12 isolate, into *C. quinoa* and *T. expansa* leaves. Necrotic spots were observed, 7 d.p.i., on *C. quinoa* leaves inoculated with P32 and P32 Δ3, otherwise chlorotic spots were observed on all other samples. *T. expansa* leaves, inoculated with P32 and P32 Δ6, showed chlorotic spots surrounded with a necrotic ring. All other constructs did not induced symptoms under visible light; however, spots appeared under UV light observation on all inoculated leaves confirming virus replication and cell-to-cell movement in *T. expansa* (Fig. III.5; Fig. III.6).

No signal was revealed on western blot analysis from extracts containing deleted forms p32 Δ4 or p32 Δ5 tagged with Flag or HA at C-terminal (data not shown). However, using anti Flag conjugate with peroxidase, we observed specific signal at 16 kDa on leaves inoculated with p32 Δ6 carrying a deletion of both N- and C-termini. Necrotic spots analysis of sample

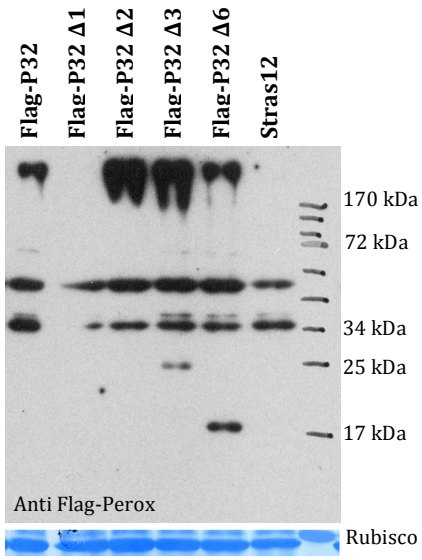

Fig. III.7 Immuno dectection of expression products tagged with flag epitope by western blot analysis. Anti Flag conjugate with peroxidase was use at 1:10,000. Coomassie brilliant blue stain was use for revealed Rubisco protein.

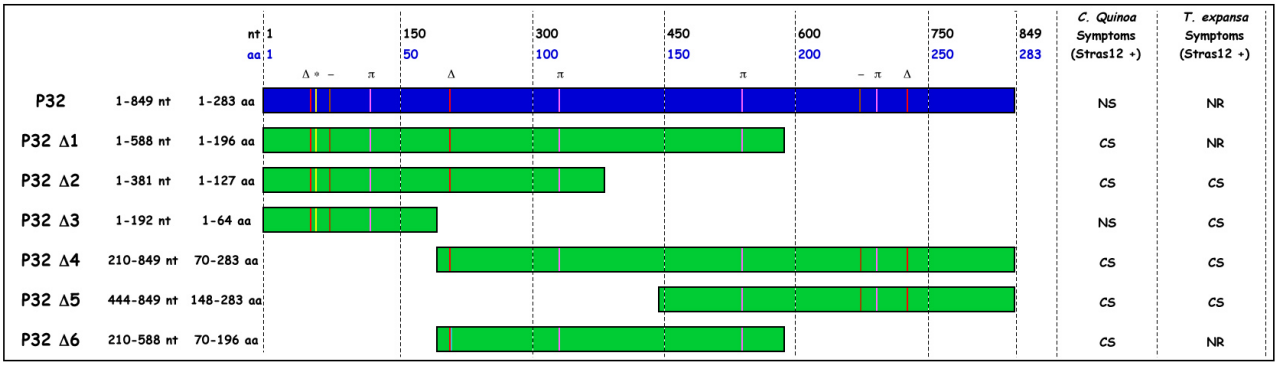

Fig. III.6 P32's deleted forms with correspond *C. quinoa* and *T. expansa* leaves symptoms.
NS: necrotic spots. CS: chlorotic spots. NR: necrotic ring spots.
Blue bar represents wild type P32; Green bars represent p32 deleted forms. Prosite motif are shown: Δ N-glycosylation site (red line); * Casein kinase II phosphorylation site (yellow line) ; - Protein kinase C phosphorylation (brown line); π Myristoylation site (purple line).

inoculated with P32 Δ3 showed a specific signal of about 25 kDa instead of the predicted 7 kDa (Fig. III.7). These results suggested that the first 64 amino acids of P32 were responsible for the induction of the necrotic spots on *C. quinoa* leaves and that maybe the P32 protein is glycosylated or myristoylated on N-terminal part in the predicted site, respectively, ^{18}NVTG^{21} (M1) or ^{69}NVSV^{72} (M2) and ^{39}GVLCNI^{44} (M3).

Transcripts corresponding to single and double mutants, on which glycosylation or myristoylation sites have been removed (Table III.7), were mechanically inoculated with Stras12. Double mutations of two glycosylation predicted sites (M1 + M2) or of glycosylation and myrostoylation (M1 + M3) prediction sites mutation as well as, single mutation on myristoylated predicted site (M3) prevent the appearance of necrotic spots (7 d.p.i.) on *C. quinoa* leaves. Western blot analysis of all lesions were performed and revealed that protein of about 32 kDa can be detected in all mutated p32 forms as well as p32 wild type. Moreover, high molecular weight products are less present for the mutated p32 forms that induced chlorotic spots on *C. quinoa* leaves (Fig. III.8).

Sample	Clone	Construction	Symptoms on *C. quinoa*
A	MD75	Rep3-FlagP29	CS
B	MDS1	Rep3-FlagP32	NS
C	MD150	Rep3-p32 N18G	NS
D	MD151	Rep3-p32 N68G	CS
E	MD157	Rep3-p32 N18G N69G	CS
F	MD168	Rep3-p32 G37V-G39V	CS
G	MD169	Rep3-p32 N18G-G37V-G39V	CS
H	MD100	Rep3-FlagP32 Δ6	CS

Table III.7 Summary of P32 mutated construction with correspondent leaves symptoms on *C. quinoa* inoculated with Stras12 isolate. NS: necrotic spots. CS: chlorotic spots.

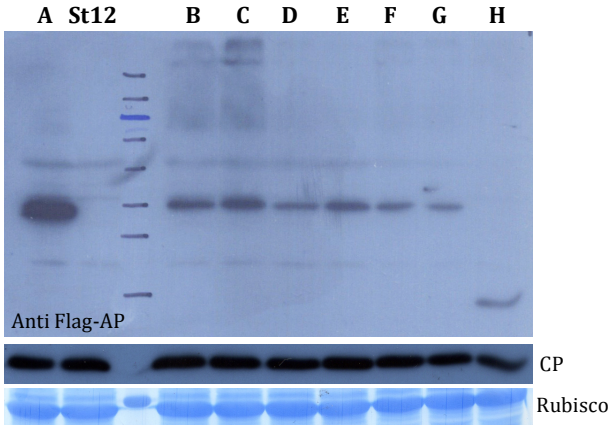

Fig. III.8 Western blot analysis of *C. quinoa* leaves inoculated with different P32 mutated forms. Order sample is report on table III.7 Anti Flag conjugate with alkaline phosphatase and anti BNYVV CP conjugate with peroxidase were use at 1:7500 and 1:60,000 dilution, respectively. Coomassie brilliant blue stain was use for revealed Rubisco protein

3.4 Subcellular localization

Transient expression vectors, based on pBIN61 plasmid ((Dunoyer et al., 2002); (Voinnet et al., 2000)), were produced to drive the expression of full-length or mutated forms of p32 protein fused to GFP at N- or C-terminals (Table IV.8). Plasmids were transformed into *Agrobacterium tumefasciens* and subsequently agroinfiltrated, using different concentration (0.3, 0.6 and 1.2 OD_{600}), into *N. benthamiana* leaves. Fluorescent cells were analysed by CLSM after 24, 48, 72 and 96 h.

Clone	Construction	Mutation
MD105	pBIN-GFP:p32	---
MD106	pBIN-p32:GFP	---
MD223	pBIN-p32N18G-G37V-G39V:GFP	M1 and M2
MD224	pBIN-GFP:p32N18G-F37V	M1 and M2
MD225	pBIN-p32N18G-N69G:GFP	M1 and M3
MD226	pBIN-GFP:p32N18G-N69G	M1 and M3

Table III.8 GFP fusion with wild type or mutated BSBMV p32 in pBIN61-based clones. M1 and M3 are mutation on first and third predicted N-glycosylation site. M2 is mutation on first predicted N-myrostoylation site..

No fluorescent cells were observed on collected samples with the exception on positive controllo using GFP alone..

A binary plasmid expressing a strong gene silencing suppressor as P19 from *Tomato bushy stunt virus* (TBSV) was then co-agroinfiltrated with previous described pBIN61-based clones expressing p32 and its mutated forms. Fluorescent cells observed on *N. benthamiana* leaves evidenced that the 59 kDa fusion proteins (GFP-p32 or p32-GFP) localized preferencially in the nucleus after 72 h. After 96 h fluorescent aggregates were observed outside nucleus close to membrane cell walls and was somehow correlated with cell death. No differences were observed, in term of subcellular localization, between P32's mutants and wild-type (Fig. III.9).

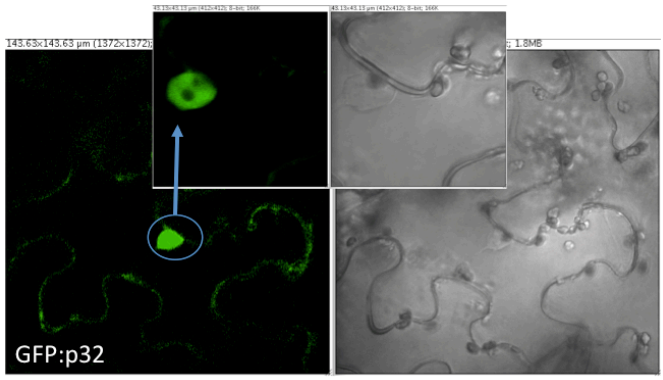

Fig. III.9 Subcellular localization of GFP:p32 protein.

Importantly, inoculation of *C. quinoa* leaves with Stras12 + tRep3-GFPp32 or tRep3-p32GFP produced necrotic spots similar to those produced by wild-type BSBMV RNA-4, Rep3-FlagP32 or Rep3-p32, confirming that the presence of GFP at N- or C-terminal didn't interfered with this p32 "activity" *in planta*.

3.5 p32 protein over expression

Genscript rare codon analysis tools (http://www.genscript.com/cgi-bin/tools/rare_codon_analysis) have been used to investigate presence of rare codon in P32 or P29 nucleotide sequence. Results evidence same Codon Adaptation Index (CAI) for both proteins (0.72) with similar GC content (42.3 and 43.2%, respectively).

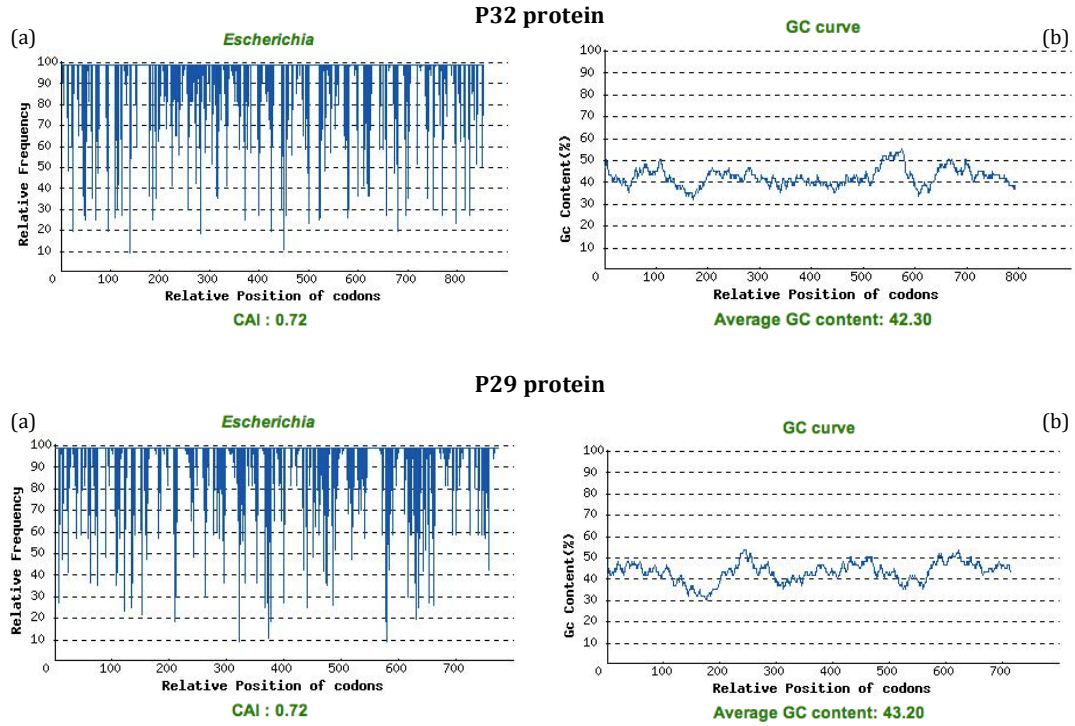

Fig. III.10 Genscript rare codon analysis. Top part: p32 nucleotide sequence; low part: p29 nucleotide sequence. (a) Distribution of codon usage frequency along the length of p29 and p32 protein sequence. In general, a Codon Adaptation Index (CAI) of 1.0 is considered to be ideal. Low numbers correspond to high chance for the gene to be poorly expressed. (b) The ideal percentage of GC content is between 30 – 70%.

In vitro expression were performed using TNT Coupled Reticulocyte Lysate Systems (Promega) with ^{35}S(methionine) according to the manufacturer's protocol using 1 µg of Rep3-FlagP32, Rep5-P32 or pUC47 (full-length BSBMV RNA-4 cDNA) plasmids and Luciferase cDNA as positive control. No protein expression has been revealed by SDS-Page

and Western blot analyses of both p32 clones or full-length BSBMV RNA-4 with the exception of well-expressed luciferase gene (Fig. III.11).

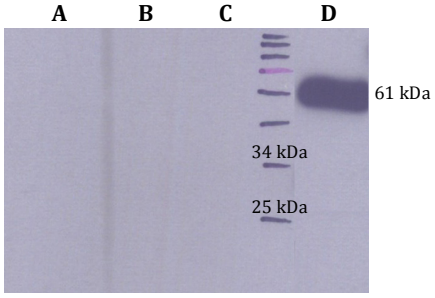

Fig. III.11 SDS-Page and western blot analysis of *in vitro* expression using TNT Coupled Reticulocyte Lysate Systems with ^{35}S(methionine). Samples: (A) Rep3-Flag-P32; (B) Rep5-P32; (C) pUC47; (D) Luciferase.

FlagP32 or P32 proteins were not successfully expressed *in vivo* even using several *E. coli* strains (Table III.9). Different protein expression conditions were also changed as growing temperature (14°C for 8 hours; 25°C or 30°C for 4 hours), increasing time after expression induction (1, 2, 3, or 4 hours) and final concentration of IPTG (0.1 mM, 0.5 mM or 1 mM). Excepted halo tagged proteins (see below), no specific expression of tagged p32 protein has been observed in *E. coli*. However, BNYVV p42 protein expression using pET42 plasmid, as well as BSBMV p29-HA expression using Rep3 viral vector, have been produced in all conditions.

Protein	Tag	Tag position	Viral vector	*E. coli* strain	WB
P32:HA	HA	N-terminal	Rep3	KRX or BL21	–
HA:P32	HA	C-terminal	Rep3	KRX or BL21	–
HA:P32:HA	HA	N- and C-terminal	Rep3	KRX or BL21	–
Flag:P32	Flag	N-terminal	Rep3 or pET	BL21	–
His:P32	His	N-terminal	pET	BL21	–
GST:P32	GST	N-terminal	pET	BL21	–
Halo:P32	Halo	N-terminal	pFN18K	KRX	+
P29:HA	HA	C-terminal	Rep3	KRX or BL21	+
Halo:P29	Halo	N-terminal	pFN18K	KRX	+

Table III.9 Heterologous tag proteins of BSBMV P32 and P29 express on *E. coli* KRX and BL21(DE3) pLysS strain. Western blot analysis were performed using specific antibody to molecular tag. P29 protein didn't show difficult expression but only Halo tag permitted to expressed P32 on *E. coli*.

As reported in Table III.9 Halo-p32 and Halo-p29 fused proteins were successfully expressed in KRX *E. coli* strain (Promega) using MD214 and MD215 clones according to HaloTag7 Technology protocols (Promega). The highest efficiency for Halo-p32 and Halo-p29 expression was obtained growing KRX E. coli strain at 25°C for 2 and 4 hours, respectively. Protocol described by Leammli et al. (1970) and freeze-thaw treatment were compared for proteins extraction from *E. coli* cells and proteins were then analysed by

SDS-page and Western blot using Anti HaloTag polyclonal antibody (Promega). Both extraction treatments allowed the detection of Halo-p29 protein but only freeze-thaw prevented the degradation of Halo-p32 (Fig. III.12). Halo-p32 was then successfully express in *E. coli* KRK strain as a soluble protein as well as Halo-p29 protein. Further experiments will aim to purify the protein to raise specific antibodies and thus obtain a tool for the study of p32 without any tag in viral context.

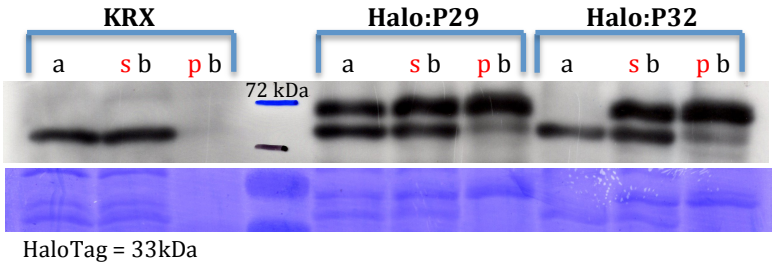

Fig. III.12 Western blot analysis of Halo-p32 and Halo-p29 proteins expression. Different extraction protocols were used: (a) Laemmli protocol; (b) Freeze-thaw treatment of pelleted cells on TE buffer, in this case supernatant solution (s) was separated from pellet (p) (Appendix xxx). Anti HaloTag polyclonal antibody was used at 1:10,000 dilution. HaloTag increases the masses of the peptides by a 33 kDa.

3.6 Yeast two hybrid

Yeast one and two-hybrid tests were performed to test if p32 was able to respectively self activate the transcription (as BNYVV p26, LINK et al or BSBMV p29, Ratti unpublished) and was able to interact with itself. Two different yeast strains were used: Y187 carrying pGAD424-derived vectors and HF7c carrying pGBT9-derived vectors. After mating, diploids were selected on Leu-Trp-depleted minimal medium and equal numbers of selected clones were plated on Leu-Trp-His-depleted (-LTH) minimal medium to test for their ability to activate the *his3* gene.

Fig. III.13 Two hybrid system analysis using pGAD-proteins and pGBT9-proteins clones transformed on yeast. Diploids expressing full length or deleted form of p32 were selected on minimal medium depleted of tryptophan and leucine (-TL) (a) and tested for the activation of the *His3* reporter gene by growth on minimal medium depleted of tryptophan, leucine and histidine (-TLH) (b). (c) Schematic representation of mating yeast clones.

The absence of growth in histidine depleted media let us conclude that p32 protein was not able to self-activate the transcription nor interact with itself in such systems (Figure III.13).

3.7 Is P32 an RNA silencing suppressor?

Guilley et al. (2009) recently published an expression system based on BNYVV replicon that allows the rapid identification of RNA silencing suppressor protein (SSP). BNYVV RNA-1 and Rep3-p30 clone carrying *Tobacco mosaic virus* (TMV) P30 ensure viral replication and cell-to-cell movement, respectively. SSP candidate gene, expressed by Rep5 viral replicon, induces necrotic spots on inoculated *C. quinoa* leaves if possess silencing suppressor activity. No symptoms appear if no SSP is present in the inoculum.

tRep5-p32 and tRep5-p31 were separately inoculated with t15 (transcripts of BNYVV RNA-1) and tRep30 (transcripts of Rep3-p30). 7 d.p.i. no symptoms were observed on

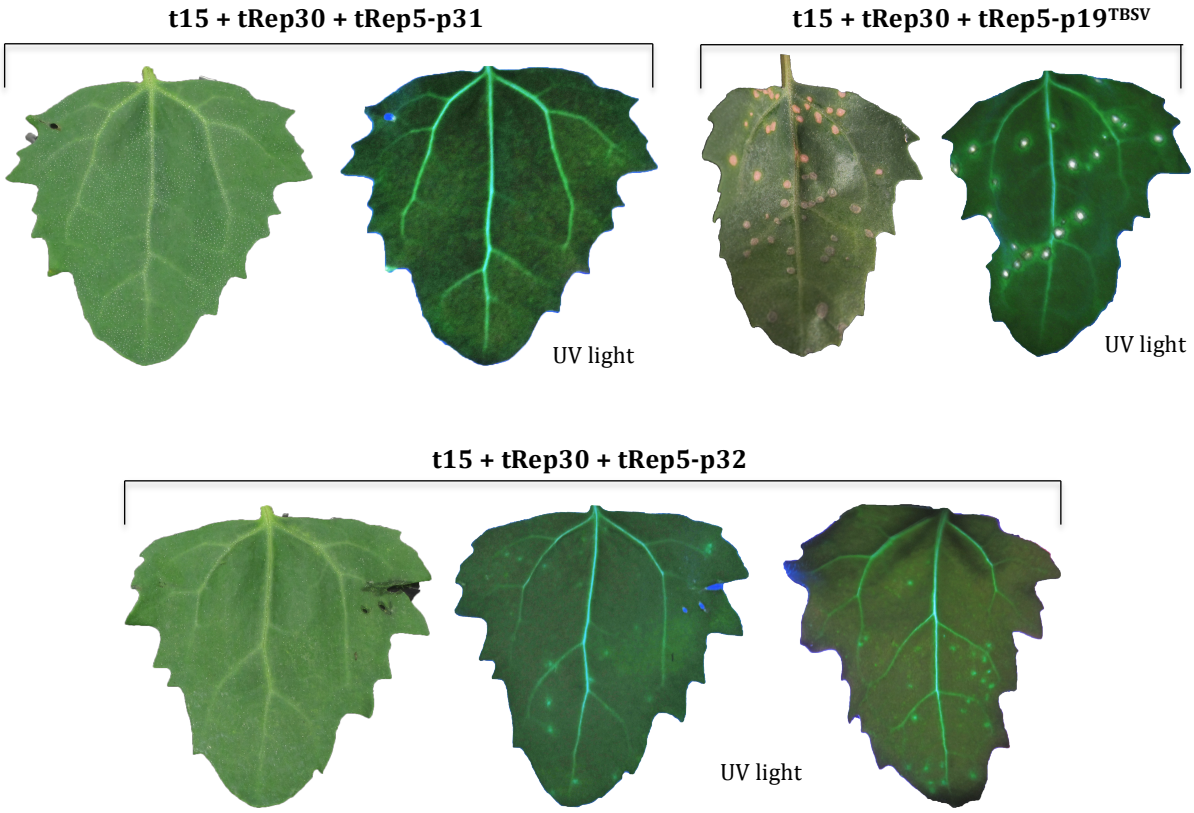

Fig. III.15 *C. quinoa* leaves symptoms showed 7 d.p.i. on visible light or under UV light. t15 transcripts of BNYVV RNA-1; tRep30: transcripts of p30 from *Tobacco mosaic virus* (TMV) into Rep3; tRep5-p31 transcripts of Rep5-p31; tRep5-p32 transcripts of Rep5-p32.

inoculated *C. quinoa* leaves under visible light but in the present of p32 protein, small chlorotic spots were observed under UV light not present when p31 protein was expressed (Fig. III.15).

Such results suggest that both proteins do not behave as silencing suppressor in leaves. The presence of small necrosis within leaves inoculated with vectors expressing p32 is probably due to an HR reaction of the host.

Chapter 4

Discussion

Discussion

Beet necrotic yellow vein virus (BNYVV) is responsible for Rhizomania disease that spread worldwide over the last 30 years. Many studies were performed to understand pathogenic mechanisms and select resistant sugar beet cultivars but high selective pressure conditions made possible independent evolution of resistance breakdown (Acosta-Leal et al., 2010). On the other hand, *Beet soil-borne mosaic virus* (BSBMV) is not involved in severe disease symptoms and until now has been reporter only in USA. BSBMV shares many characteristics with BNYVV such as vector, host ranges, particles morphology and genomic organization. However, the viruses are not serologically related (Lee et al., 2001). Interestingly, BNYVV helper strain (RNA-1 and -2) is able to replicate and encapsidate BSBMV RNA-3 (Ratti et al., 2009).

Up to date not many studies have been performed on BSBMV. The aim of my PhD was to develop reverse genetic tools for the study of BSBMV in order to improve knowledge on *Benyvirus* pathogenic mechanisms and transmission. We successfully obtained, for the first time, full-length infectious cDNA clone for all BSBMV RNAs, starting from an infested soil carrying viruliferous BSBMV *P. betae*. Sequence analyses (Chapter 1) revealed nucleotide substitutions between our BSBMV MRM06 isolate genome and previously-characterized EA isolate. Both shared high nucleotide identity scores except for RNA-4 that was unexpectedly longer and carrying and unknown ORFs organization (Table I.7).

One of the major criteria for the classification of virus species is complementation between RNA segments from two RNA viruses with divided genomes (Van Regenmortel et al., 1997). We showed that BNYVV RNA-1 or -2 and BSBMV RNA-2 or -1 could form a chimeric virus, using infectious *in vitro* transcripts from full-length cDNA clones, confirming strong correlation between this two viral species.

Malyshenko and co-authors (1989) reported complementation between different related and unrelated plant viruses for cell-to-cell spread when they were pre-inoculated on leaves. Complementation is common for movement protein or coat protein and can be observed between different viruses species, as *Cucumber mosaic virus* (CMV) and *Tomato aspermy virus* (TAV) that can exchange their RNAs-3 (Salánki et al., 1997), or genus, as *Cymbidium mosaic virus* (CymMV) a Potexvirus, that are complemented with *Odontoglossum ringspot virus* (ORSV) a Tobamovirus (Ajjikuttira et al., 2005). On *Benyvirus* genus, Bleykasten-Grosshans and co-authors (1997) evidenced complementation of

BNYVV triple gene block (TGB) proteins when TGB proteins were expressed by RNA 3-derived replicon (Rep3), in combination with RNA-2 mutants that were defective for the corresponding gene. P42 and p13 expressed by Rep3, for instance, were able to complement the corresponding defective RNA-2 mutants but Rep3-p15 was unable to provide complementation of the p15-defective RNA-2, resulting in inhibition of cell-to-cell movement and virus replication. According to our results complementation between TGBp2 (p13) and TGBp3 (p15) of BSBMV and BNYVV occurs when BNYVV p13-p15 proteins were expressed by Rep3 and co-inoculated with BNYVV RNA-1 and p13-p15 defective BSBMV RNA-2 (EUB22) or p15-defective BSBMV RNA-2 (MD231). Moreover our analysis revealed that expression of BNYVV p15 by Rep3 can complements p15-defective BSBMV RNA-2 mutant in the presence of BNYVV RNA-1 transcripts, inducing chlorotic spots on inoculated *C. quinoa* leaves. It has been previously proposed that full-length and truncated forms of BNYVV p15 could compete for binding sites on another component (which could be of either viral or cellular origin) involved in the movement process (Bleykasten-Grosshans et al., 1997). We demonstrated that such competition doesn't occurs when defective BSBMV p15 (carried by BSBMV RNA-2) was complemented by BNYVV p15 (expressed by Rep3) suggesting similar activity but different targets between BSBMV and BNYVV p15 proteins. As cell-to-cell movement is a crucial action for virus infection, our observation suggests a new research line that can be developed to discover biological mechanisms on which Benyviruses infectivity is based in order to identify possible strategies to protect sugar beet plants.

Complementation between BSBMV and BNYVV RNAs is also supported by experiments conducted on a new full length form of BSBMV RNA-4 identified during my PhD research activity. The new BSBMV RNA-4 shares 47 % nucleotide sequence identity with BNYVV RNA-4, instead of 55% identity of BSBMV RNA-4 described by Lee and co-authors (2001), and is replicated and encapsidated by BNYVV helper strain (RNA-1 and -2). Moreover we demonstrated for the first time the essential role of BSBMV RNA-4 on vector transmission confirming similarity with BNYVV RNA-4 functions. Previous studies showed that wild-type (wt) BNYVV RNA-4 is required for efficient transmission of BNYVV by the plasmodiophorid *P. betae* (Tamada and Abe, 1989). Its presence in the inoculum produced strong chlorotic lesion in *T. expansa* leaves and leaf stunting, curling and severe mosaic with leaf distortions on *N. benthamiana*. BNYVV p31 plays a multifunctional role in efficient vector transmission, enchanced symptom expression and root-specific silencing suppression, moreover, BNYVV RNA-4 deleted forms, encoding truncated p31 protein, are not able to transmit the virus (Rahim et al., 2007). Up to date BNYVV p31 function have

been deduced from experiments performed using full-length or mutated forms of BNYVV RNA-4, therefore maintaining coding sequence in the natural context it wasn't possible to exclude influence of UTRs sequence on p31 function. During my research I have also expressed BSBMV p32 and BNYVV p31 proteins by Rep5 viral vector, demonstrating, for the first time, that encoded proteins alone can support BNYVV RNAs (1, 2 and 3) transmission through *P. betae*.

During our experiments a spontaneous deletion mutant of RNA-4 appears during serial mechanical inoculation and it isn't able to transmit virus through *P. betae*. As previous reported internal deletions or loss of smaller RNA species have been described for BNYVV isolates maintained by repeated mechanical inoculation of local lesion hosts (Hehn et al., 1994). Prolonged cultivation of field-infected plants, especially when growth at high temperatures results, induced spontaneous deletion of part of *Soil-borne mosaic virus* RNA-2 (Chen et al., 1995). Our results then suggest that BSBMV RNA-4 previous described (Lee et al., 2001) is a spontaneous deleted form.

In our experiments full length BSBMV RNA-4, as well as, wt or tagged p32 protein expressed by Rep3 with BNYVV helper strain, have been associated, on *C. quinoa* leaves, to an induction of necrotic spots. Moreover yellow spots with necrotic ring appeared on *T. expansa* leaves inoculated with BSBMV RNA-4.

Western blot analysis identified a specific high molecular weight products on sample inoculated with FlagP32. Absence of P32 self-interaction in yeast suggests that high molecular weight product is the results of some interactions with other viral/plant protein(s) or could be mediated by post translational modifications.

Domains of p32 involved on necrotic symptoms are probably located on the N-terminal part of the protein as deleted forms p32 Δ4 to Δ6 are not able to induce necrosis on *C. quinoa* (Fig. III.5 and III.6). In particular p32 Δ3 form induced necrotic symptoms and presented an electrophoresis mobility shift and high molecular weight products on western blot analyses suggesting the presence of post translational modifications sites located within the N-terminal part of the protein.

High molecular weight products specifically disappeared when glycosylation (M2) or myristoylation (M3) predicted sites, present within the N-terminus of p32 was altered. Such data suggests that these post transcriptional modifications are essential for p32 functionality *in planta* as confirmed by absence of necrosis on symptoms induced by M2 and M3 mutants (Table III.7 and Fig. III.8).

In contrast, mutation of myristoylation predicted site apparently doesn't affect the subcellular-localization of the p32 protein which mainly accumulates into the nucleus

within the first 72h. The existence of such nuclear localization suggests the absence of myristoylation modification that is supposed to enhance protein -membrane or protein-protein interactions (Podell and Gribskov, 2004). Moreover, after 96h GFP:p32 or p32:GFP proteins re-localized into the cytoplasm, forming fluorescent aggregates close to the membrane that were observed before cell death. According to our observation and bibliographic studies, we emitted the hypothesis that p32 myristoylation occurs, post-translationally, when internal glycine residues become exposed by cleavage during apoptosis associated to necrotic spots (Zha et al., 2000).

According to this hypothesis we cannot exclude that, in the presence of *P.betae* vector, p32 protein follows a different metabolic pathway where interaction with protozoa protein prevents its myristoylation. This aspect will need a better investigation when specific p32 antibody will be available. The use of p32 deleted forms and punctual mutants will be tested for transmission in order to establish a possible correlation between the domain involved in the transmission and necrosis induction.

As no antibody against p32 is available at the moment, during our researches we successfully expressed in *E. coli* KRX strain soluble p32 tagged with Halo protein. This important result will allow us, in the closely future, to produce and purify enough protein to obtain polyclonal antibody.

It is know that viral proteins associated with symptom severity often show silencing suppression activity. As for BNYVV p31, (Rahim et al., 2007) a possible activity of p32 as a suppressor of RNA silencing cannot be excluded. A rapid screening of p32 RNA silencing suppressor activity has been performed according to the protocol developed by Guilley et al. (2009). No silencing suppression function was evidenced in such preliminary experiments. We noticed the induction of visible spots under UV light that may correlate with an hyper-sensible response. Here again, further studies are necessary to evaluate a similar activity in a root context.

In *B. macrocarpa*, BNYVV long distance movement is related to the presence of RNA-3 and involves a nucleotide sequence named “core” (Lauber et al., 1998). Recently, a conserved ”coremin” sequence has been identified within core motif of BNYVV and BSBMV RNAs-3, in BNYVV RNA-5 and BSBMV RNA-4 as well as in other viral species belonging to the genus *Cucumovirus.* The presence of two motifs within BSBMV (RNA-3 and -4) was indicated as one possible explanation for less efficient complementation of BSBMV RNA-3 for long-distance movement of BNYVV RNA-1 and -2 in *B. macrocarpa* plants compared to

BNYVV RNA-3 (Ratti et al., 2009). We demonstrated that presence of a second coremin sequence is not able to influence, positively or negatively, the long-distance movement efficiency. This result supports the hypothesis that another *cis* or a *trans* element on BSBMV RNA-1 and/or -2 could have an effect on long distance movement. Such element is yet unidentified.

RNA recombination is a process that joins non-contiguous RNA segments together that plant RNA viruses are often subjected to. Viral RNA recombination is thought to occur when the viral replicase accidentally switches templates during complementary RNA synthesis ((Lai, 1992); (Nagy and Pogany, 2000)).

The resulting novel combinations of genes, sequence motifs, and/or regulatory RNA sequences could cause dramatic changes in the infectious properties of RNA viruses that can potentially lead to the emergence of new viruses or strains. Therefore, RNA recombination can help viruses to escape natural resistance mechanisms contributing to viral outbreaks (Cheng et al., 2006).

As described above, appearance of BNYVV RNA-3 and -4 deleted forms has been reported after successive mechanical inoculation to *C. quinoa* leaves (Bouzoubaa et al., 1991) but, to our knoledge, no natural recombinant RNAs (Chimeras) have been described on viral species belonging to genus *Benyvirus*. In this paper we therefore report for the first time a natural chimeric RNA derived from recombination process between BSBMV RNAs -4 and -3 that occurred during serial mechanical inoculation on *C. quinoa* leaves. Study of the chimeric RNA influence on BSBMV fitness was not part of the present study otherwise sequence analysis revealed two putative ORFs encoding small proteins which could be important for BSBMV adaptability. Interestingly, we demonstrated the ability of chimeric RNA to escape, during replication process, from competition with BNYVV or BSBMV RNA-3 (data not shown). This characteristic makes chimeric RNA the best candidate to become the basis of a new viral vector able to ensure co-expression of 3 different foreign proteins when used together with previous described Rep3 or RepIII and Rep5 replicons ((Bleykasten-Grosshans et al., 1997);(Schmidlin et al., 2005); (Ratti et al., 2009)).

Chapter 5

Conclusion

Conclusion

In conclusion my PhD thesis gained important knowledge about *Benyvirus* genus. First, we developed full-length infectious clones of all four BSBMV RNAs, confirming their infectivity *in planta* and their replication and encapsidation on BNYVV helper strain context. Moreover we described an unknown BSBMV RNA-4 demonstrating that RNA-4 previous described is a deleted form that presumably can't support virus transmission through the vector. Using *P. betae* we also demonstrated that full-length BSBMV or BNYVV RNA-4 encoded proteins, p32 and p31 respectively, can support virus transmission of BNYVV RNAs (1, 2 and 3) through the vector into their natural contest as well when expressed by Rep5 viral vector. Analysis of BSBMV p32 protein revealed a complex expression profile with putative glycosylation, myristoylation and/or host protein(s) interaction that need to be better investigate in the future. Finally our research products ensure the opportunity to investigate, in the near future, domains of BSBMV p32 protein and/or BNYVV p31 protein essential for virus transmission and then to detect host protein(s) involved on transmission opening a new strategy to develop resistant sugar beet plant toward Rhizomania disease.

Appendix

Material and methods

Virus infection

BNYVV RNA

C. quinoa leaves (usually 6 to 8 weeks old) have been mechanical inoculated with 10 µl of a solution containing BNYVV RNA-1 and -2 from *in vitro* transcription (50 µg/ml of each transcripts), 50 mM of KH_2PO_4 and 0.04% of macaloid using Celite as abrasive powder on the leaves. Seven days after inoculation, symptomatic leaves are been harvested and total RNA extracted. RNA solution, containing BNYVV RNA-1 and -2 called Stras12, has been used to inoculate other plants (*C. quinoa*, *T. expansa*, *B. vulgaris*, *B. macrocarpa* ecc.) in the presence or not of one or more viral genomic RNAs or viral vectors (Rep3, Rep5 or RepIII) synthesized by *in vitro* transcription.

In vitro transcription

This strategy is widely used to produce viral single-stranded RNAs from DNA clones. Two conditions are required to allow efficient transcription:

a) a transcription promoter should be upstream the DNA sequence that must to be transcribed and, in the present study, T7 promoter from phage (TAATACGACTCACTATAGGG) was chosen;
b) during run-off transcriptions, DNA should be linearized where transcription has to stop , in the present study this point coincide with the end of poly-A tail.

The RiboMAX™ Large Scale RNA Production System – T7 (Promega) kit was used to synthesize RNAs of interest, following manufacturer protocol. DNA template (1 µg) was mixed with 4 µl transcription buffer 5x-concentrated, 1.5 µl of each rATPs (100 mM), rUTPs (100 mM), rCTPs (100mM), 0.06 µl of rGTPs (100 mM), 0.5 µl of RNAseOUT (40 U/µl) (Promega), 1 µl of $m^7G^{5'}ppp^{5'}$ CAP analog (Promega), 2 µl of T7 enzyme mix and sterile water up to 20 µl final volume. After incubation at 37°C for 30 minutes, 1.5 µl of rGTPs (100 mM) was added to the reaction mix that was then left for 3 h 30 min at 37°C to complete transcription. Belated addition of rGTPs in the reaction mix favours the incorporation of CAP analogs in the newly synthesized transcripts. The transcription efficiency is checked by running a non-denaturating 1x-TBE agarose gel with 1 µl of each transcribed sample.

Mechanical inoculation

Leaves of different host plants (*C. quinoa, T. expansa*, *B. macrocarpa*) were mechanical rub-inoculated with viral RNA and/or transcribed RNAs. Each leaf was dusted with Celite to promote mechanical lesions and facilitate penetration of RNAs into plant cells and then rubbed gently with 5 to 15 µl (according to leaves size) of inoculation solution composed by 10 µg of each RNAs transcripts, 5 µg of BNYVV RNA-1 and -2 (Stras12), 10 µl of potassium phosphate buffer (0,5 M KH_2PO_4 pH 7.5), 8 µl of macaloid 0.5% and sterile water up to 100 µl. After 7 days lesions from inoculated leaves were recovered and used for total RNAs or proteins extraction.

Agroinfiltration

Agroinfiltration is an efficient methods for transient expression of gene in plants. Agrobacteria (*Agrobacterium tumefaciens*) were cultured for 18 h at 28°C in 5 ml of liquid LB selective medium (10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl in 1 L final volume) supplement with rifampicin (100 µg/ml) and appropriate antibiotic to select binary plasmid, in these thesis pBIN61 vector was selected with 50 µg/ml of kanamycin. Bacteria were centrifuged for 15 min at 5,000 g and pellet was resuspended in infiltration buffer (10 mM $MgCl_2$, 200 µM acetosyringone) adjusting the $OD600_{nm}$ to 0.6. Bacteria were then incubated at room temperature for 3 hours to allow transfer activation. Four leaves of *Nicotiana benthamiana* plants were lightly incised with a scalpel then infiltrated with the bacterial suspension using a syringe without a needle. The maximum expression of the gene carried by pBin61 vector was usually reached between 2 to 5 days post-infiltration, leaves were then harvested for analysis and / or observation by confocal microscope.

RNA and DNA processing

RNA extractions

Working on plant RNA viruses, extraction of total RNAs from fresh infected plant material is essential to deal with the viral genome. This was performed using two different protocols:

a) Trizol® total RNA extraction (Invitrogen)
b) TM extraction method (Jupin *et* al., 1990) (Mapping sequence)

Trizol total RNA extraction

To extract RNA from infected *C. quinoa*, *B. macrocarpa* or *T. expansa* leaves and *B. vulgaris* roots, the Trizol® total RNA extraction were followed according to manufacturer's protocol. Fresh or frozen leave or roots (100-200 mg) were crushed in a sterile 1.5 ml eppendorf with 1 ml of Trizol buffer. After 5 min at room temperature, 200 µl of chloroform are added and tubes are vigorously shaken by hand and again incubate at room temperature for 2 min. After centrifugation for 15 min at 12,000 g and 4°C, the aqueous phase is transfer to a fresh tube where is added 0.5 ml of isopropanol. RNA precipitation requires an incubation for 20 min at room temperature, then the RNA is pelleted by 12,000 x *g* for 30 min at 4°C, and washed with 1 ml of 70% ethanol. RNA pellet is dried 5 min on air and then resuspended in 20 µl of sterile water. RNAs quality and quantity are analysed on agarose gel and by spectrophotometer, respectively.

Viral encapsidation test

To test if RNA is packaged into a viral particle, two RNA extractions are performed in parallel: a Trizol RNA extraction (described above) and a Tris-$MgCl_2$ buffer, called TM protocol (Jupin et al. 1990). Extraction with TM buffer can keep only the encapsidated RNA. Fresh or frozen leaves or roots (50 – 100 mg) were crushed in 500 µl TM buffer (100 mM Tris-HCl, 10 mM $MgCl_2$ pH 7.5). The homogenate was incubated at 37°C for 30 min to leave RNases present in the extract to degrade free RNA then cellular debris was eliminated by low-speed centrifugation. Purification by phenol and phenol-chloroform-isoamyl alcohol (25:24:1) were performed. The supernatant was removed and the RNAs were precipitated with 150 mM $NaCl_2$ and two volume of 100% ethanol. The pellet obtained was resuspend in 200 µl of 3 M sodium acetate pH 5.6 and incubate for 30 min at -20°C. After centrifugation at 13,000 g for 30 min at 4°C, the pellet was washed with 70% ethanol. RNA pellet is dried 5 min and then resuspend in 20 µl of sterile water. RNAs quality and quantity are analysed on agarose gel and by spectrophotometer, respectively.

RNA-DNA amplification and visualization

Polymerase chain reaction (PCR) is a powerful methods that permit to generate millions of DNA copies, from a limited amount of nucleic acid, usable for direct visualization or further manipulations. While DNA is immediately suitable for such amplification, RNA must be reverse transcribed.

Reverse transcription

The reverse transcription step aims to synthesize for each RNA of interest the anti-complementary DNA strand, also called cDNA. This last is then amplifyied by PCR. Two reverse transcriptase were used in the present study.

Moloney murine leukemia reverse transcriptase (M-MLV RT) (Promega) was used for the common production of short fragments (up to 1-2 kb). RNA samples, mixed with 1 µl reverse primer (25 µM) and nuclease-free water up to 5 µl final volume, is first heated during 10 min at 65°C in a T3000 Thermal Cycler (Biometra) to disrupt secondary structures. The elongation step is performed at 37°C during 1 h after the addition of 4 µl of 5x-concentrated buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$ and 50mM DTT), 2 µl dNTPs (10 mM), 0.25 µl M-MLV RT (200 U/µl) and 3.75 µl steril water nuclease-free.

When high quantity of longer cDNA fragments (1.5 – 6.0 kb) needed to be synthesized, ImProm-II Reverse Transcriptase (Promega) was preferred. The first denaturation step is identical to that described for M-MLV RT then were added 4 µl ImProm-II 5x-reaction buffer, 1.2 µl $MgCl_2$ (25 mM), 1 µl dNTPs (10 mM), 0.5 µl RNasin Ribonuclease Inhibitor (40 U/µl), 1 µl Improm-II RT and 7.3 µl nuclease-free water. Elongation is performed during 60 min at 42°C followed by RT inactivation at 70°C for 15 min.

Polymerase chain reaction (PCR)

Two types of thermostable DNA polymerases synthesizing dsDNA were used in the presented experiments. Go Taq® Flexi DNA polymerase (Promega) was used to amplify 1-2 kb fragments that didn't require high accuracy of copying, e.g. viruses detection or clones screening. 5 µl of cDNA from RT step is mixed with 5 µl Go Taq® Flexi buffer 5x-concentrated, 2.5 µl $MgCl_2$ (25 mM), 0.75 µl dNTPs (10 mM), 1 µl of each primer forward and reverse (10 µM), 0.12 µl of Go Taq® DNA polymerase (5 U/µl) and sterile water up to 25 µl final volume. When DNA fragments were amplified for subsequent cloning or sequencing, Pfu Ultra II Fusion Hotstart Polymerase (Agilent Technologies Italia) was preferred. Because this last includes a proof-reading Pfu DNA polymerase, the amplification accuracy is threefold higher compared to systems using only the first enzyme. The reaction mix is as follow, 5 µl PfuUltra II reaction buffer 10x-concentrated (containing $MgCl_2$), 1 µl of dNTP mix (25mM each dNTP), 1 µl of each forward and reverse primer (10 µM), 1 µl *PfuUltra* II Fusion HS DNA Polymerase, 5 µl cDNA from reverse transcription, finally volume is adjusted with nuclease-free water to 25 µl.

Separation of DNA fragments on agarose gel by electrophoresis

DNA molecules, whether coming from PCR reaction or other protocols (see after), may be separated according to their size. For such purpose, gels are prepared by melting Multi Purpose agarose (Roche) in 1x-concentrated Tris-Borate-EDTA buffer (TBE, 89 mM Tris-borate, 8.9 mM boric acid and EDTA 2 mM) to concentrations varying between 0.7 and 2.0%, depending on the fragments size. Using Biorad Power Pac 300 or Modell 1000/500 power supply (Biorad), DNA fragments are subsequently forced to migrate through the gel in TBE buffer 1x-concentrated towards the anode, as being negatively charged. After running, gel is incubate on 200 ml of Ethidium bromide solution (0.1 mg/ml) that allow double strand DNA visualization under UVs. Using 1 kb or 100 bp DNA ladders (Promega), the approximate size of the observed fragments can be determined.

Purifications of nucleic acids

DNA and RNA molecules may be purified in the view of further manipulations. Three types of purification methods were used in this work, depending on the nature of the molecules (DNA or RNA) and the aim of the purification step.

When a single-type/length of DNA had to be selected among molecules of different sizes, all fragments were separated on an agarose gel and the fragment of interest was selected by cutting it out from the gel. DNA was subsequently extracted from the piece of agarose by using affinity columns of Wizard SV gel and PCR clean-up system (Promega) and eluted with nuclease-free sterile water.

In order to eliminate salts and chemicals present in solution with DNA from a previous reaction and make the DNA clean for further applications, or just for concentrate DNA after gel extraction a phenol-chloroform method was preferred. Equal volumes of hydrophobic phenol:chlorofom:isoamyl alcohol (25:24:1, pH 4.5) solution and DNA are mixed and centrifuged 15 min at 14,000 g at 4°C. The upper aqueous phase containing DNAs molecules is then precipitated with two volumes of 100% ethanol, 16 µl NaCl (5 M) and 1 µl glycogen (10 mg/ml) at -20°C during 20 min. After 20 min centrifugation at 14,000 g at 4°C, the pellet is washed with 70% ethanol, dried and resuspended in nuclease-free sterile water.

If RNA needed to be purified, the phenol-chloroform method was preferred followed by RNAs precipitation with two volumes of 100% ethanol and sodium acetate (150 mM final concentration) at -20°C for 1 h. RNAs was then pelleted, washed, dried and resuspended as described for DNA.

Cloning

Cloning vector and expression

pGEM®-T Easy (Promega). It is a 3 kb linear vector with thymines overhanging at 3' extremities. These prevent the plasmid from recircularization and facilitate the insertion of PCR products with 5' adenines overhangs, added by classical polymerases with no proofreading activity. T7 and SP6 transcription promoters are also included on both sides of the insertion site that is inside a multiple cloning site (MCS) with numerous restriction sites. pGEM®-T Easy vector is widely used for common cloning of PCR products and contain ORF of ampicillin resistance gene that is used on selective medium. In addition, MCS of this vector is situated inside another gene coding for the β-galactosidase. If disrupted by insertion of a PCR product, the enzyme is not able to degrade the X-galactose anymore and bacteria appear white on Petri dishes, instead of blue colony.

pUC19 (Fermentas). It is a 2.7 kb circular vector and should be open by means of restriction treatment (see after) in the MCS to insert the DNA sequence of interest. The simple arrangement of this vector makes it easy to work with, as it doesn't contain specific transcription promoters near the cloning site and its MCS is composed of many different restriction sites. It has *amp*^{R} gene (ampicillin resistance gene) and MCS, as for pGEM®-T, is located inside N-terminal fragment of β-galactosidase (*lac Z*). Moreover we modified pUC19's MCS using specific primers that allow us to insert four restriction enzyme sites: *Not*I and *Spe*I at 5'-terminal of MCS; *Mlu*I and *Bgl*II at 3' terminal of MCS. We used this plasmid to obtain full-length cDNA clones of BSBMV RNAs adding T7 promoter to 5'-terminal of virus cDNA sequence.

pGBT9 (Clontech). This 5.5 kb vector was used to highlight transcription activation activity of viral protein on yeast one-hybrid system methods. It carries a gene for ampicillin resistance, used for its selection in *E. coli* and the selection gene *TRP1*, nutritional marker that allow yeast auxotrophs carrying pGBT9 to grow on limiting synthetic medium lacking tryptophan (Trp). Proteins ligate on MCS (in this study we used *Eco*RI and *Sal*I restriction enzyme sites) are fuse to the GAL4 DNA-binding domain (Gal4 DNA-BD) present in this plasmid. Fusion proteins are expressed in yeast host cells from the constitutive alcohol dehydrogenase (ADH) promoter. The fusion protein is imported into the nucleus by nuclear localization sequences (NLS) that are an intrinsic part of the Gal4 DNA-BD.

pGAD424 (Clontech). This 6.6 kb vector was used to highlight the protein-protein interactions between viral proteins in yeast two-hybrid system methods. It carries a gene for ampicillin resistance, used for its selection in *E. coli* and the selection gene *LEU2*, nutritional marker that allow yeast auxotrophs carrying pGAD424 to grow on limiting synthetic medium lacking leucine (Leu). Proteins ligate on MCS (in this study we used *Eco*RI and *Sal*I restriction enzyme sites) are fuse to the GAL4 activation domain (Gal4 AD) present in this plasmid. The fusion proteins are expressed in yeast host cells from the constitutive alcohol dehydrogenase (ADH) promoter. The fusion protein is imported into the nucleus by nuclear localization sequences (NLS) that are an intrinsic part of the Gal4 AD.

pGEX-2TK (Pharmacia Biotech). This vector derived from pGEX-2T encodes the GST (glutathione-S-transferase). It contains a cloning cassette for cloning the gene of interest in N-terminal fusion with glutathione S-transferase (GST). The protein fused to GST was expressed in *E. coli*. These fusion proteins can be cleaved by thrombin to separate GST and the protein of interest. GST is under the control of the tac promoter which is inducible by the lactose analog isopropyl β-D thiogalactoside (IPTG). This vector also contains the gene that encodes a lacIq repressor capable of binding to the tac promoter, thus blocking the expression of the protein fused to GST until induction by IPTG.

pET. The pET plasmid (Studier et al., 1990) derived from pBR322 was used to overexpress protein of interest in *E. coli*. It confers resistance to ampicillin. The vector used contains the sequence coding of BSBMV p32 protein tagged with HA or Flag at N- or C-terminal. The sequence of interest is cloned downstream of the T7 phage promoter recognized by T7 RNA polymerase.

pFN18K (Promega). This vector is specifically design to overexpress protein of interest fuse at N-terminal with HaloTagT7® in *E. coli* or in cell-free translation systems. It confers resistance to kanamycin. Between HaloTagT7 and protein of interest is present TEV protease site that is used during purification of the protein. The lethal Barnase gene will be deactivated only when protein of interest is insert within *Sgf*I and *Pme*I restriction enzyme sites.

pBIN61 (Voinnet *et al.*, 2000). This vector is derived from pBin 19 (Bevan, 1984). Its T-DNA borders delimited by RB and LB contains the gene for resistance to kanamycin, NPT II coding for neomycin phosphotransferase and a cloning cassette flanked by the 35S promoter and terminator of CaMV 35S. This vector has an origin of replication BHR (Broad Host Range) for replication both in *E. coli* and *A. tumefaciens*.

Plasmid and insert processing

To obtain the desired final construct with the sequence of interest in a specific vector, DNA often needed to be processed.

A-tailing

When PCR products were amplified using the Pfu Ultra II Fusion HotStart Polymerase, the proof-reading activity of *Pfu* enzyme removed 5'-A overhangs from amplicons. For an efficient cloning of the amplified fragments in a vector with 3'-T overhang, adenines are added at 5' extremities of DNA fragments before cloning. The PCR product is therefore purified, mixed with 1 µl Go Taq polymerase (5 U/µl), 1 µl 10x-concentrated buffer, 0.5 µl dNTPs (10 mM) and 1 µl $MgCl_2$ (25 mM) in a final volume of 10 µl, and incubated at 72°C for 30 min.

Restriction enzyme

When DNA fragments were transferred from pGEM-T Easy to pUC19 vector or directly inserted into this last or other vector used in this thesis, treatments with restriction enzymes were necessary before ligation (see below). Restriction enzymes recognize specific DNA sequences – commonly of 6 nt, but sometimes smaller or longer – and cleave the double-strand to produce two cohesive or protrusive, 3' or 5', extremities. 1 µg of template DNA is incubated with 0.4 µl of restriction enzyme (10 U/µl) and the appropriate restriction buffer at the temperature required by the enzyme (mostly 37°C) for 3 hours. Digested fragment(s) are analysed and purified on agarose gel as described above.

Dephosphorylation

To avoid self-ligation of the restricted plasmid and favour the insertion of the DNA of interest, phosphate groups from 5'-extremities of the linearized vector may be removed. As phosphate groups are necessary for the ligation of DNA strands, the fragment to be inserted brings the only phosphate groups available and insertion is the only way to get circularization. 1 µg of linearized vector is mixed with 1 µl of alkaline phosphatase from calf intestine (20 U/µl) (Roche), 2 µl dephosphorylation buffer 10x-concentrated (0.5 M Tris-HCl, 1 mM EDTA pH 8.5) and water to 20 µl final volume. The reaction is performed at 37°C for 60 min.

Ligation

All ligation reactions were performed using the Rapid DNA ligation kit (Fermentas) according to the manufacturer's protocol. To optimize cloning conditions, a one to three ratio of vector/insert as well as the length of both DNA fragments determine the quantity of insert to add to the ligation reaction, following the formula:

$$qty\ of\ insert\ to\ clone\ (ng) = \frac{vector\ qty\ (ng)\ x\ insert\ length\ (kb)}{vector\ length\ (kb)}\ X\ \frac{3}{1}$$

Usually, 1 µl of vector (50 ng/µl), 3 µl of insert (50 ng/µl), 3 µl Rapid DNA ligation buffer 5x-concentrated, 1 µl DNA ligase (1 U/µl) and nuclease-free sterile water up to 15 µl, were used for reaction, follow incubation for at least 1 hour at room temperature. After phenol:chloroform extraction and precipitation with ethanol, the ligation products are resuspended in 3 µl of nuclease-free sterile water and 1.5 µl are used for electroporation.

Site direct mutagenesis by "overlap extension" PCR

Different kind of sequence modifications may be introduced in constructs.

Site-directed PCR mutagenesis, involves the extension of overlapping fragments by PCR amplification (Ho et al., 1989). As the primers are the ends of newly-synthesized strands, a mutation can be engineered during binding of DNA template strand in the first cycle of a PCR reaction. This technique requires four oligonucleotide primers, two of which (inside amplicon fragment) are mutated and two stages of PCR amplification. Primers mutated have a complementary part containing overlapping region, near the mutation that we want to introduce. In the first step, two PCR amplifications were performed with primer pairs 1 + 3 and 2 + 4 respectively. The two DNA fragments, which have a common region, are purified to remove the plasmid template. Purified products are used as template in the second step of PCR amplification with primers 1 + 4. The final product, usually digested by two restriction enzymes, can be inserted into a vector digested with the same enzymes.

Detection of viral components

Three different techniques were used to detect viral RNAs or proteins, depending on what had to be evidenced.

RT-PCR

Multiplex RT-PCR, aiming to detect simultaneously *P. betae*, BNYVV, BSBV, BVQ, and BSBMV was developed by Ratti (Ratti et al., 2005) and co-authors in 2005 and used in the present work as a common tool to identify RNAs extracts from sugar beet roots or *C. quinoa* leaves. However, RT-PCR proved to be too sensitive when working with full-length infectious clones and the following Northern and Western blot techniques were therefore preferred.

Northern blot

Viral RNAs were detected by Northern blot in RNAs extracted from samples infected with full-length infectious transcripts. Extractions were performed following the Trizol protocol (Invitrogen, described above).

Electrophoresis

Total RNAs samples are separated according to their size on an agarose gel composed of TBE 1x-concentrated, 1% melted agarose and 6% formaldehyde that ensures denaturation of migrating RNAs. For each sample, 5 µg RNAs are mixed with four volumes of RNA loading buffer (20mM HEPES-KOH pH 7.8, 1mM EDTA, 50% formamide, 6% formaldehyde, 0.005% bromophenol blue, 0.001% ethidium bromide and 3.7% glycerol) before loading in the gel's wells. Migration is performed under the hood in HEPES buffer 1x-concentrated (20 mM HEPES, 1 mM EDTA and 17 mM KOH) at 50-80 mAmperes depending on the gel size.

Blotting and fixation

After gel migration, RNAs are transferred on a nylon Hybond-NX membrane (GE Healthcare) by capillarity with a high salt SSC 20x solution (3 M NaCl and 0.3 Sodium Cytrate). After transfer RNAs are fixed on the membrane by exposition to 254 nm-UVs, 120 µJoule/cm^2 with Stratalinker® UV Crosslinker (Stratagene). The transfer efficiency is

then checked by visualization of cellular RNAs, as ethidium bromide from RNA loading buffer makes them visible under UVs.

Probes synthesis and purification

Viral RNAs are detected on the nylon membrane through hybridization of specific radiolabeled probes, which sequences are anti-complementary to a limited part of the targeted RNAs. 30 ng of purified PCR fragments (220-750 nts) are denatured at 100°C for 2 min in 20 µl nuclease-free sterile water, transferred on ice and then mixed with 12 µl H_2O, 10 µl Labeling buffer 5x-concentrated (Promega), 2 µl Bovine serum albumine (BSA) (10 mg/ml), 2 µl d(AGT)TPs PCR Grade (500 µM each) (Roche) and 1 µl DNA Polymerase Klenow (5 U/µl) (Promega). 25-30 µCi [α_{32}-P]dCTP (3000 Ci/mmol) are added to each reaction tube for production of radiolabelled probes. After incubation for 60-90 min at room temperature, reactions are stopped by heating at 100°C for 2 min and then directly chilled on ice. Finally, probes are purified on Illustra MicroSpin™ G-25 Columns (GE Healthcare) according to manufacturer's protocol.

Hybridization, washing and exposition

Membranes with cross-linked RNAs are placed in tubes with PerfectHyb™ Plus Hybridization Buffer (Sigma) inside a Hybridization Oven/Shaker (Amersham). During pre-hybridization step (1 h at 60°C) RNA-free sites of the membranes become saturated by the buffer. Probes have been added in the tubes and left for O/N for hybridization step at 55°C. On the following day, buffer from the tubes is collected (probes may be used several times) and membranes are washed four times at 65°C for 15 min with 2x SSC with 0.1% sodium dodecylsulfate (SDS) buffer (2 times) and then 0.2x SSC with 0.1% SDS buffer (2 times). Dried membranes are left with an Amersham Hyperfilm ECL (GE Healthcare) for 20 min to one week exposure periods, depending on the radioactivity emitted by radiolabeled RNAs. Films are revealed manually.

Western blot

Samples preparation

Two different samples preparation methods were followed:

a) Protein extraction from plant material

Inoculated tissues that need to be analysed for the presence of viral proteins, are directly crushed in 2x Laemmli buffer (125 mM Tris-HCl pH 6.8, 20% glycerol, 5% SDS, 0.01% bromophenol blue, 5% β-mercapatoethanol) (Laemmli, 1970). Leaves or single lesion are crushed with 2-3 ml or 50 µl of Laemmli buffer, respectively, homogenate by vortexing boiled for 3 min at 95°C to denature proteins then centrifuged for 5 min at 13,000 g to pellet cellular debris.

b) Protein extraction from bacteria

Protein expression on *E. coli* were performed as described below. After expression, the cultures are centrifuged for 10 min at 5,000 g. Pellet was resuspended in 2 ml of sterile water and separated in two 1.5 ml tubes and centrifuged for 5 min at 13,000 g. Pellet in one tube was directly resuspended into 1x Laemmli buffer (sample a on Fig. III.12) and pellet in the second tube was resuspended into 100 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA 7.5 pH) and subjected to a 3 cycles of freeze-thaw process into nitrogen liquid for 1 min followed by 2 min at 30°C and then 100 µl of 2x Laemmli buffer were added (sample b on Fig. III.12). The proteins are then separated from cell debris by centriugation at 13,000 g for 5 min and then denatured by heating 5 min at 95°C.

Separation of proteins under denaturing conditions (SDS-PAGE)

Proteins are separated according to their molecular weight (MW) on SDS-polyacrylamide gels. However, these are composed of two superimposed gels with distinct functions. The proteins separation occurs in the lower resolving gel, which polyacrylamide concentration is adapted to the MW of the proteins to be separated. Bigger proteins require low concentration of polyacrylamide whereas high-concentrated gels are used for smaller ones. The upper stacking gel is prepared at low polyacrylamide concentration and is crucial for the proteins concentration before entering in the resolving gel. The stacking gel consists of 4% acrylamide, 0.1% bisacrylamide, 125 mM Tris-HCl pH 6.8, SDS 0.1%. The resolving gels used in this thesis are 8, 12 or 15% concentrated (8-15% acrylamide, 0.2-0.37% bisacrylamide, 275 mM Tris-HCl pH 8.8, 0.1% SDS). The final addition of 0.05% TEMED and 0.1% ammonium persulfate catalyses the polymerization of acrylamide with

bisacrylamide between two glass plates. The electrophoresis is performed at 80 V until samples reach the resolving gel and then 100 V to the end in appropriated running buffer (25 mM Tris-Base pH 8.3, 192 mM glycine, 0.1% SDS).

Immunodetection of proteins by Western Blot

The principle of this technique is to detect the presence of a protein after transfer to a membrane, using a specific antibody. Proteins transfer from polyacrylamide gel to membrane ImmobilonTM (MilliporeTM) is performed in a system Mini-Protean/Trans-Blot (Bio-Rad®) at 80 V for 1 h 30 min at 4 ° C in 25 mM Tris-HCl pH 8.3 and 192 mM glycine. The membrane is then pre-incubated for 30 min in PBS-Tween 1% (137 mM NaCl; 100 mM Na_2HPO_4; 2 mM KH2PO4; 2.7 mM KCl; 1% Tween) supplemented with 5% skimmed milk before adding the primary antibody at appropriate concentration. After overnight incubation at 4 ° C, the membrane is washed 3 times, 20 min each, with PBS-Tween 1%, then incubated for 3 h in PBS-Tween 1% + skimmed milk 5% with the secondary antibody coupled to peroxidase. The revelation was made after 3 washes of the membrane with PBS-Tween 1%, by autoradiography with the Lumi-Light kit (Roche®). Primary antibody rabbit polyclonal antibody (pAb) specific to BNYVV or BSBMV CP are used at 1:60,000 dilution. Rabbit anti-Flag polyclonal IgG (Sigma-Aldrich) is used at 1:7,500 dilution and anti-Halo pAb (Promega) is used at 1:10,000 dilution; as secondary antibody anti-rabbit peroxidase monoclonal antibody (Sigma-Aldrich) is used at 1:10,000 and anti-rabbit alkaline phosphatase-conjugated IgGs (Sigma-Aldrich) were used at 1:7,500 dilution. During western blot analysis we used antibody directly conjugate with peroxidase as monoclonal anti-HA-peroxidase and polyclonal anti-Flag-peroxidase (Sigma-Aldrich) at 1:20,000 and 1:7,500 dilution, respectively.

Protein expression

BSBMV p32 protein was tagged with different proteins (HA, Flag, GST, Halo) and *in vitro* and *in vivo* protein expression was performed. Two different protocol were used, TNT Coupled Reticulocyte Lysate Systems for *in vitro* expression and HaloTag Technology for expression in KRX *E.coli* strain.

In vitro protein expression

Transcription and translation reaction were performed in the same reaction starting from cDNA plasmids that contain T7 promoter sequence followed by wild-type protein fuse or not with HA or Flag, using TNT couple reticulocyte lysate systems (Promega). T7 RNA polymerase was used for transcription and radiolabelled methionine was incorporated into protein using the following protocol: 25 µl TNT Rabbit Reticulocyte Lysate, 2 µl TNT Reaction buffer, 1 µl amino acid mixture minus methionine (1mM); 1 µl [^{35}S]methionine (10 mC$_i$/ml), 1 µl RNasin Ribonuclease inhibitor (40 U/µl), 1 µl TNT RNA T7 polymerase (20 U/µl), 2 µl DNA template (0.5 µg/µl) and nuclease-free water to a final volume of 50 µl were incubated at 30°C for 90 min. Proteins were analysed following SDS-PAGE analysis protocol using 10 µl of reaction into 10 µl of Leammli buffer 2x-concentrated.

E.coli protein expression

pET and pFN18K vectors were used for BSBMV p32 protein expression on BL21 and KRX *E. coli* strains.

HaloTag® Protein Purification System was used to purify the proteins according to the protocol suggested by manufacturer (Promega).

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Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the bead of bacteriophage T4. Nature **227**, 680-685.

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Oligonucleotides

Full-length BSBMV RNA-1 cloning		
Primer	Sequence	Amplicon
BSBMV1 NotI T7	AAAGCGGCCGCTAATACGACTCACTATAGAAATTCTTC	A
	CCATTCGCCATCATTGAATCGTT	
BSBMV1 R10	AAACGCGTGAAAATGATTCGGGATCTTC	
BSBMV1 F11	AAAGCGGCCGCTCTCGATATGTTCGTGCAC	B
OLIGOdT25 Mlu R	AAAACGCGTTTTTTTTTTTTTTTTTTTTTTTTT	
BSBMV1 NotI T7 5' RACE	AAAGCGGCCGCTAATACGACTCACTATAGAAATTCGAT	C
	CTTTCCCACCCACCATCATTG	
BSBMV1 R10	AAACGCGTGAAAATGATTCGGGATCTTC	

Full-length BSBMV RNA-2 cloning		
Primer	Sequence	Amplicon
BSBMV2 NotI T7F	AAAGCGGCCGCTAATACGACTCACTATAGGGATTCTAA	D
	TTATTATCTCCATTGAATAGAA	
BSBMV2 XmaI R	CTTTCAGTCTTCCCGGGCATAC	
BSBMV2 XmaI F	GAGCGTATGCCCGGGAAGAC	E
BSBMV2 XbaI R	AAACTGCAGTCTAGACATTATCTATCCTCGCAAAAGG	
BSBMV2 XbaI F	AAATGTCTAGAGAAATAACGGCCCGTGC	F
OLIGOdT25 BglII R	AAAAGATCTTTTTTTTTTTTTTTTTTTTTTTT	
BSBMV1 NcoF	TG*CCATGG*ACCCCTACTAGG	G
BSBMV2 XmaI R	CTTTCAGTCTT*CCCGGG*CATAC	
BSBMV2 F1	TAGTAAGGCTTGCACAGGTG	H
OLIGOdT25 BglII R	AAAA*GATCTT*TTTTTTTTTTTTTTTTTTTTTTTT	
BSBMV2 NotI T7AA	AAA***GCGGCCGC***TAATACGACTCACTATAGAAATTCTAA	I
	TTATTATCTCCATTGAATAG	
BSBMV1 NcoR	AGGGGT*CCATGG*CAGATTCC	

Full-length BSBMV RNA-4 cloning	
Primer	Sequence
BSBMV4 NotI T7 F	AAA*GCGGCCGC*TAATACGACTCACTATAGAAATTCAAA
	ACTCAAAAATATAATTTTGTATTTCC
OligodT25 BglII	AAAGATCTTTTTTTTTTTTTTTTTTTTTTTT

Primer on BSBMV RNA-2 TGB proteins	
Primer	Sequence
BSBMV RNA2 p13 Mut R	TGTTGCATGAAAGCCAGCAATAC
BSBMV RNA2 p13 Mut F	GCTTTCATGCAACAAAAACATAAG
BSBMV2 p15 Mut F	GTGTTTAATGTTGTTTGTGTGG
BSBMV2 p15 Mut R	CATTAAACACACCATGAACAAAC
BNYVV p15 NcoI F	AAA*CCATGG*ATGGTGCTTGTGGTTAAAG
BNYVV p15 Bam R	AAA*GGATCC*TTATCTATGATACCAAAAC
BNYVV p13 NcoI F	AAA*CCATGG*ATGTCTAGGGAAATAAC
BNYVV p13 Bam R	AAA*GGATCC*TTAACCACAAGCACCATTAC
BSBMV p13 NcoI F	AAA*CCATGG*ATGTCTAGAGAAATAAC
BSBMV p15 Bam R	AAA*GGATCC*TTAACTATGATACCAAAAC

5' RACE PCR on BSBMV RNA-1 and -2	
BSBMV1 R 294-275	AGATCATGCTTCCAAATGGC
BSBMV1 R 195-176	CGAAACAGCAAAACCTCCAT
BSBMV2 384-365	TTGAGGGTAACTGGAAACCG
BSBMV2 293-274	TAACAGGTAAGCTTGCGGCT

BSBMV RNA-4 p32 protein and mutation	
Primer	Sequence
BSBMV Flag-p32 NcoI	AAA*CCATGG*ACTACAAGGACGACGACGACAAG**CCAGG A**GCCGATGTGGAGATTTGCCG
BSBMV p32 BamHI	AAA*GGATCC*TCACTGAAAATCTTGTTCGAAACAAAAC
BSBMV Flag-p13 NcoI	AAA*CCATGG*ACTACAAGGACGACGACGACAAG**CCAGG A**TGGATCAGTATACCCTTCCCTC
BSBMV p13 BamHI	AAA*GGATCC*TCACAAATAATCACTGAAAATCTTG
BNYVV FlagP31 XmaI F	AAA*CCCGGG*ATGGACTACAAGGACGACGACGACAAG**CC AGGA**GCTGATGGAGAGATATGTCGGTG
BNYVV p31 SalI BamHI R	AAA*GTCGACGGATCC*CTAATCGTGATAAAAGACAAAC
FS P32 F NcoI	AAA*CCATGG*ACTGGCCGAT*TGGAGATTTG
FS P32 F1	TCACTATAGATGCTGGATCAG
FS P32 R1	TATACTGATCCAGCATCTATAGTG
FS P32 F2	CCCTCTCAATGAGAATGGTTGTGC
FS P32 R2	TCATTGAGAGGGAAGGGTATACTG
FS P32 F3	ACGATTTGTGTAACGTGGTTTGGTTGGAG
FS P32 R3	ACCACGTTACACAAATCGTTGAGTGG
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BSBMV p32Flag203-212 R	AAA CCCGGG TCCTGG CTTGTCGTCGTCGTCCTTGTAGTC ACGAAAAGGACGACCGC
BSBMV p13 NcoI F	AAACCATGGGGATCAGTATACCCTTC
BSBMV p13HA PsrI BamHI R	AAACTGCAGGGATCCTCATGCATAATCAGGAACATCAT AAGGATACAAATAATCACTGAAAATC
BSBMV p32 NcoI F	AAACC ATG GCC GAT GTG GAG ATT TG
BSBMV p32 HA PstI BamHI R	AAACTGCAGGGATCCTCATGCATAATCAGGAACATCAT AAGGATACTGAAAATCTTGTTCG
BSBMV HAp32 NcoI F	AAACCATGGATGTATCCTTATGATGTTCCTGATTATGC AGGAGCCGATGTGGAGATTTG
BSBMV p32 PstI BamHI R	AAACTGCAGGGATCCTCACTGAAAATCTTGTTCG
BSBMV HAp13 NcoI F	AAACCATGGATGTATCCTTATGATGTTCCTGATTATGC AGGATGGATCAGTATACCCTTC
BSBMV p13 PstI BamHI R	AAACTGCAGGGATCCTCACAAATAATCACTGAAAATC
BSBMV p32 NcoI Flag F	AAACCATGGACTACAAGGACGACGACGACAAGCCAGG AGCCGATGTGGAGATTTGCCG
BSBMV p32to7 SalI PstI BamHI R	AAAGTCGACCTGCAGGGATCCTCACTTGTTAAGCCAAG GAAAAATATCACG
BSBMV p32to14 SalI PstI BamHI	AAAGTCGACCTGCAGGGATCCTCACACGTCAACAAGTT CATCGTGGACAAC
BSBMV p32to22 SalI PstI BamHI	AAAGTCGACCTGCAGGGATCCTCAATAGCGCCGGGGGT CACTATAAAAG
BSBMV NcoI FlagP25fromp32 F	AAACCATGGACTACAAGGACGACGACGACAAGCCAGG AGTTTCTGTTGATGTGCCAGTTAGTAG
BSBMV NcoI p25fromp32 F	AAACCATGGTTTCTGTTGATGTGCCAGTTAG
BSBMV NcoI p16fromp32 F	AAACCATGGGTTGGAGTGATGTCACTATG
BSBMV p32 EcoRI F	AAAGAATTCGCCGATGTGGAGATTTGCCG
BSBMV p32 SalI PstI BamHI R	AAAGTCGACCTGCAGGGATCCTCACTGAAAATCTTGTT CGAAACAAAAC
BSBMV p32 N18mutG R	TAACACCCAGCAAAGGTTCCTGAC
BSBMV p32 N18mutG F	CTTTGCTGGGTGTTACCGGATATG
BSBMV p32 N69mutG R	GAAACACCCATAGACGTAATCTTG
BSBMV p32 N69mutG F	CGTCTATGGGTGTTTCTGTTGATG
BSBMV p32 G36V G39V R	CACCACTAAGGGGACAATATCAATTCGCTC
BSBMV p32 G36V G39V F	TTGTCCCCTTAGTGGTGTTGTGCAATATC
BSBMV FSp32 F (NcoI)	AAACCATGGACTGGCCGATTGGAGATTTG
BSBMV FSp32 F1	TCACTATAGATGCTGGATCAG
BSBMV FSp32 R1	TATACTGATCCAGCATCTATAGTG
BSBMV FSp32 F2	CCCTCTCAATGAGAATGGTTGTGC
BSBMV FSp32 R2	TCATTGAGAGGGAAGGGTATACTG
BSBMV FSp32 F3	ACGATTTGTGTAACGTGGTTTGGTTGGAG
BSBMV FSp32 R3	ACCACGTTACACAAATCGTTGAGTGG
BSBMV p32 SgfI F	ACCGCGATCGCCATGGCCGATGTGGAGATTTG
BSBMV p32 PmeI R	ATGGTTTAAACTCATGAAAATCTTGTTCGAAACAAAAC
BSBMV p29 SgfI F	ACCGCGATCGCCATGGATTTGAATACTATGATG
BSBMV p29 PmeI R	ATGGTTTAAACTCACCATCATCTGTGTTAG
BSBMV gstP32 Bam F	AAAGGATCCGCCGATGTGGAGATTTG
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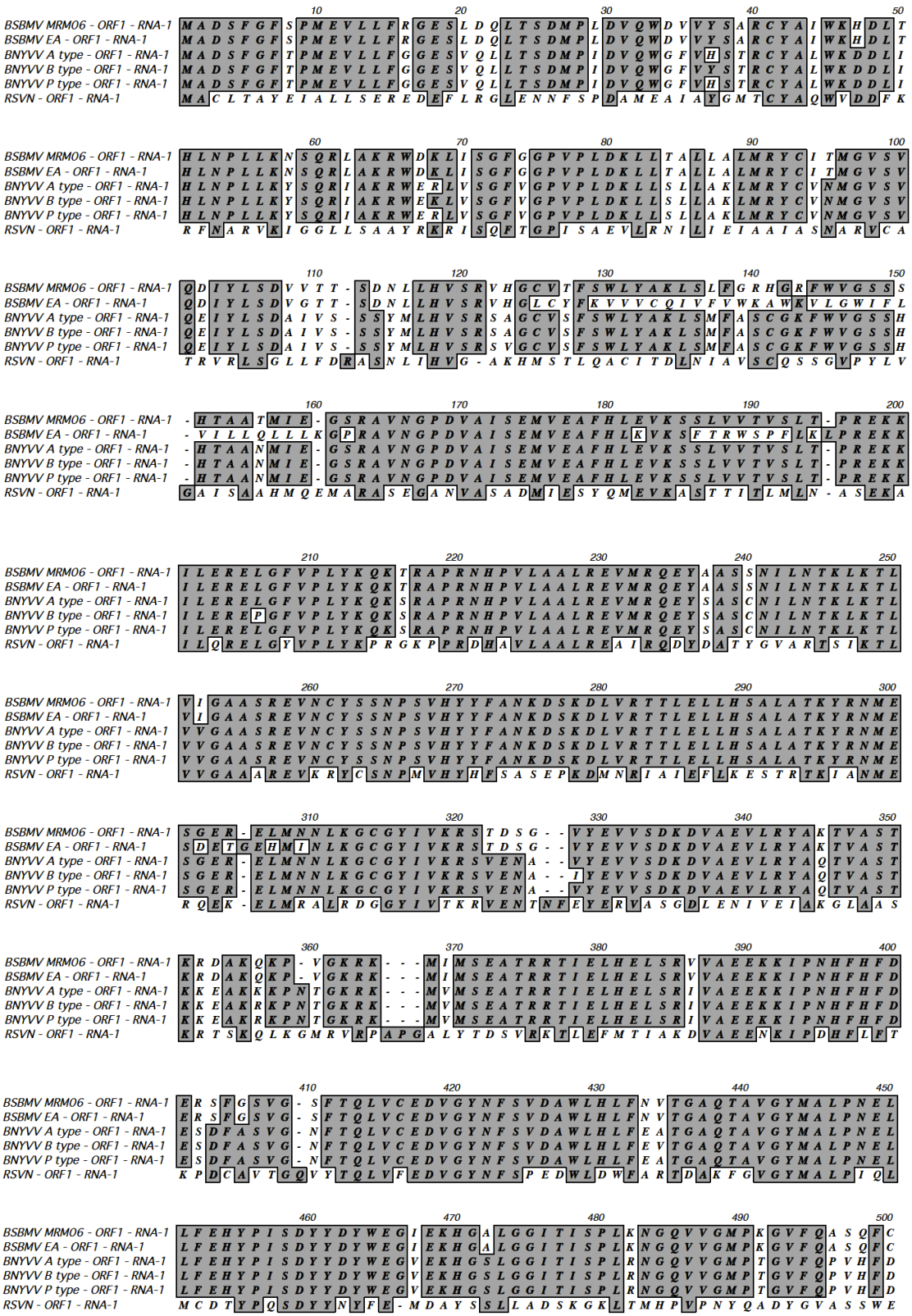
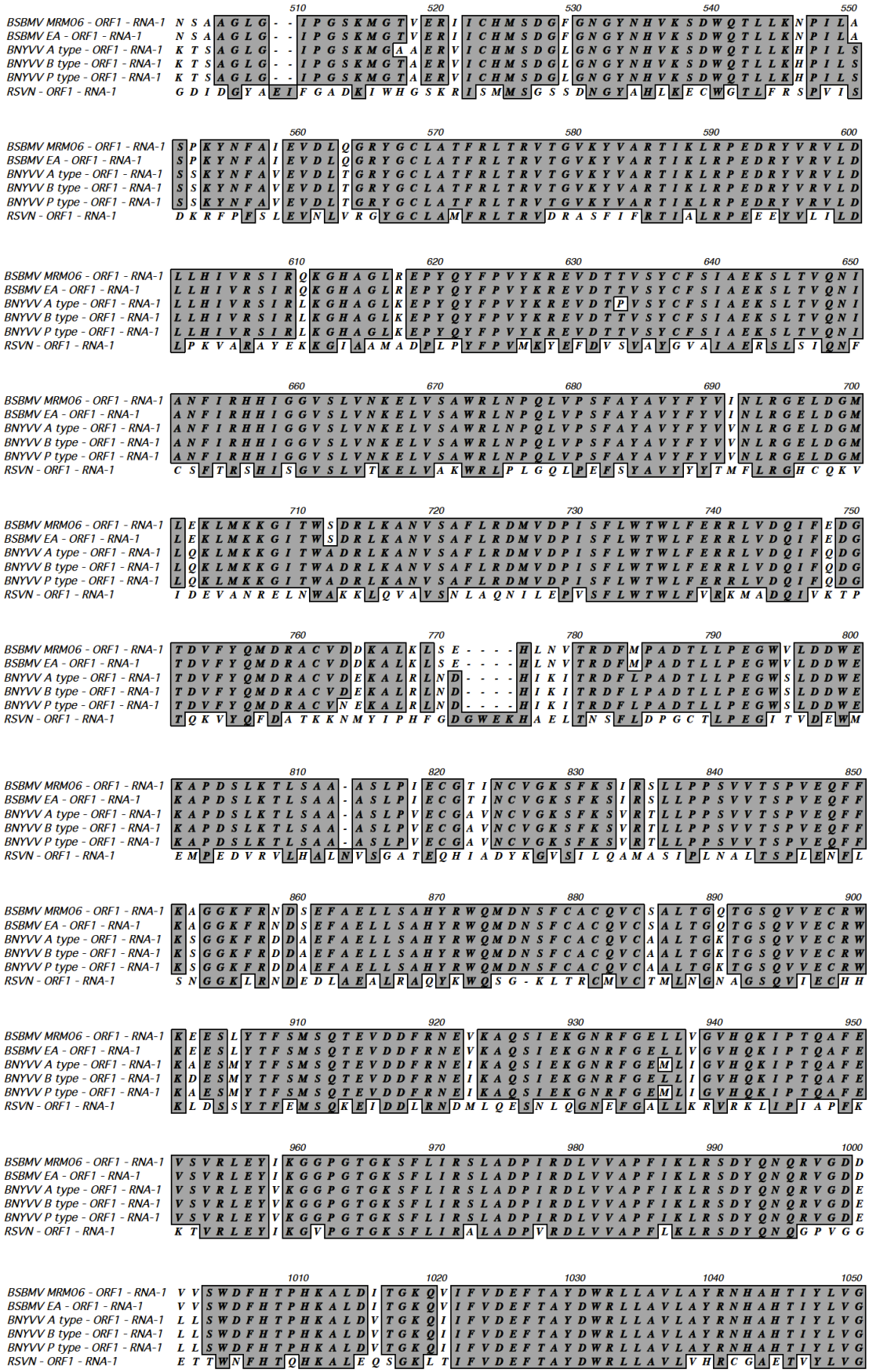
Benyvirus RNA-1 ORF1

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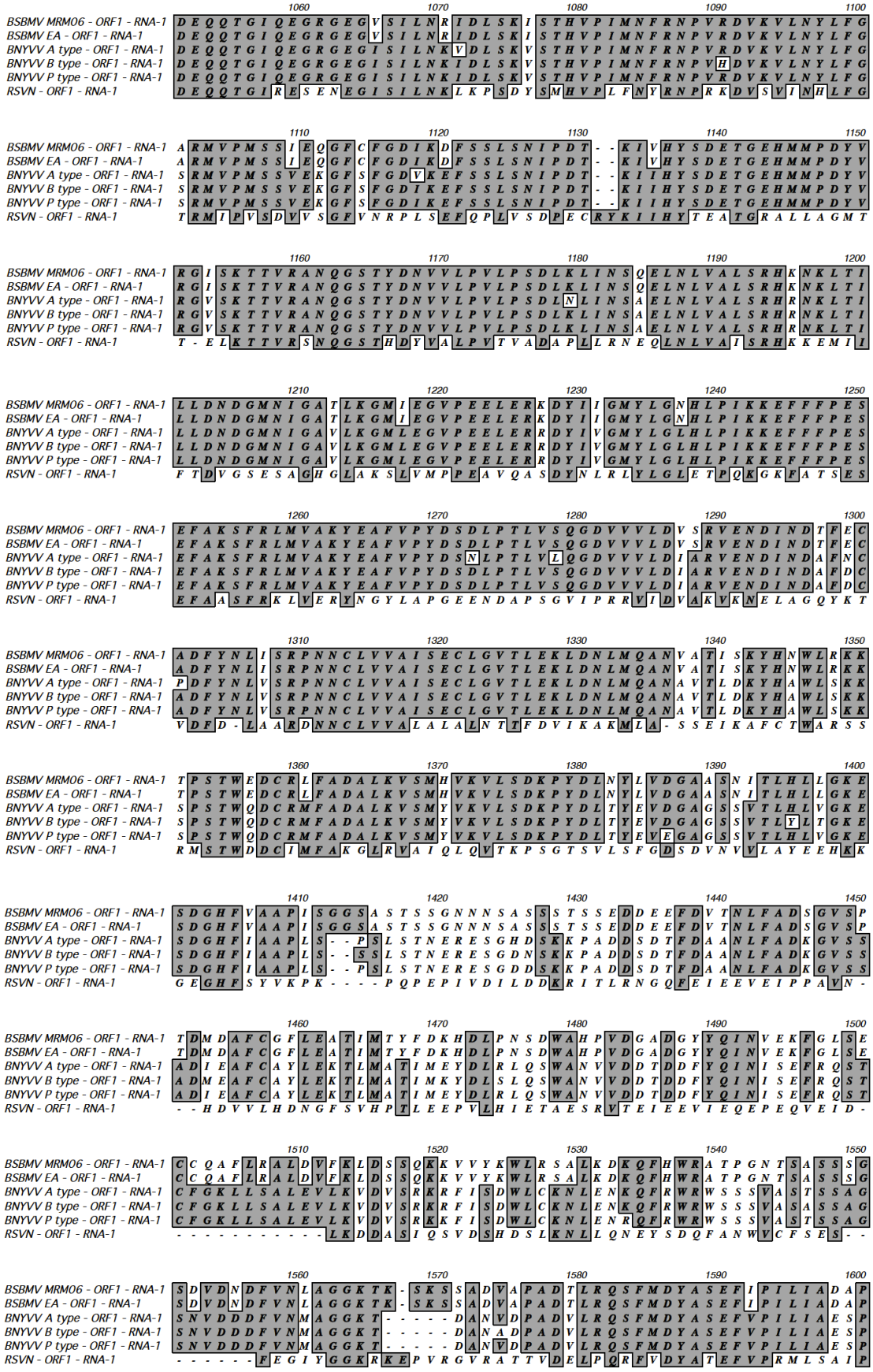
RSVN - ORF1 - RNA-1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BSBMV EA - ORF1 - RNA-1 D E Q Q T G I Q E G R G E G V S I L N R I D L S K I S T H V P I M N F R N P V R D V K V L N Y L F G
BNYVV A type - ORF1 - RNA-1 D E Q Q T G I Q E G R G E G I S I L N K V D L S K V S T H V P I M N F R N P V R D V K V L N Y L F G
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BNYVV P type - ORF1 - RNA-1 D E Q Q T G I Q E G R G E G I S I L N K I D L S K V S T H V P I M N F R N P V R D V K V L N Y L F G
RSVN - ORF1 - RNA-1 D E Q Q T G I R E S E N E G I S I L N K L K P S D Y S M H V P L F N Y R N P R K D V S V I N H L F G
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BNYVV P type - ORF1 - RNA-1 R G V S K T T V R A N Q G S T Y D N V V L P V L P S D L K L I N S A E L N L V A L S R H R N K L T I
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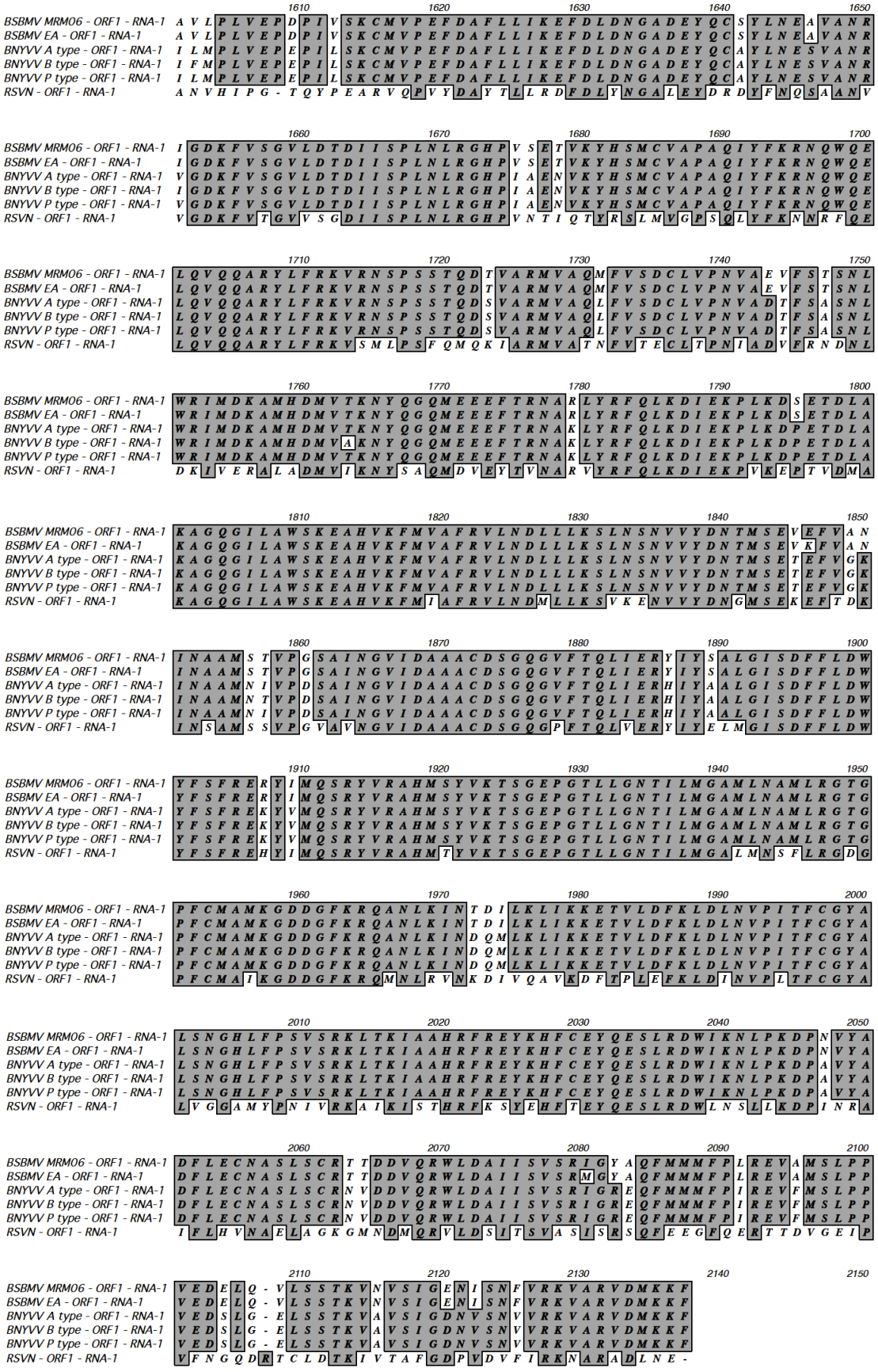
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1660 1670 1680 1690 1700
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2060 2070 2080 2090 2100
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RSVN - ORF1 - RNA-1 I F L H V N A E L A G K G M N D M Q R V L D S I T S V A S I S R S Q F E E G F Q E R T T D V G E I P
2110 2120 2130 2140 2150
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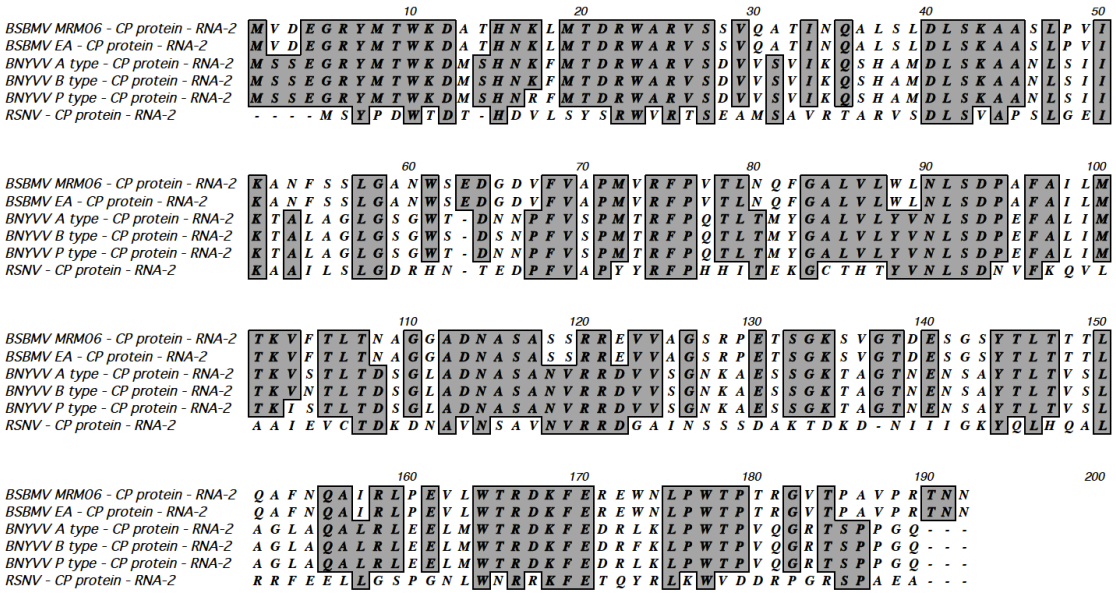
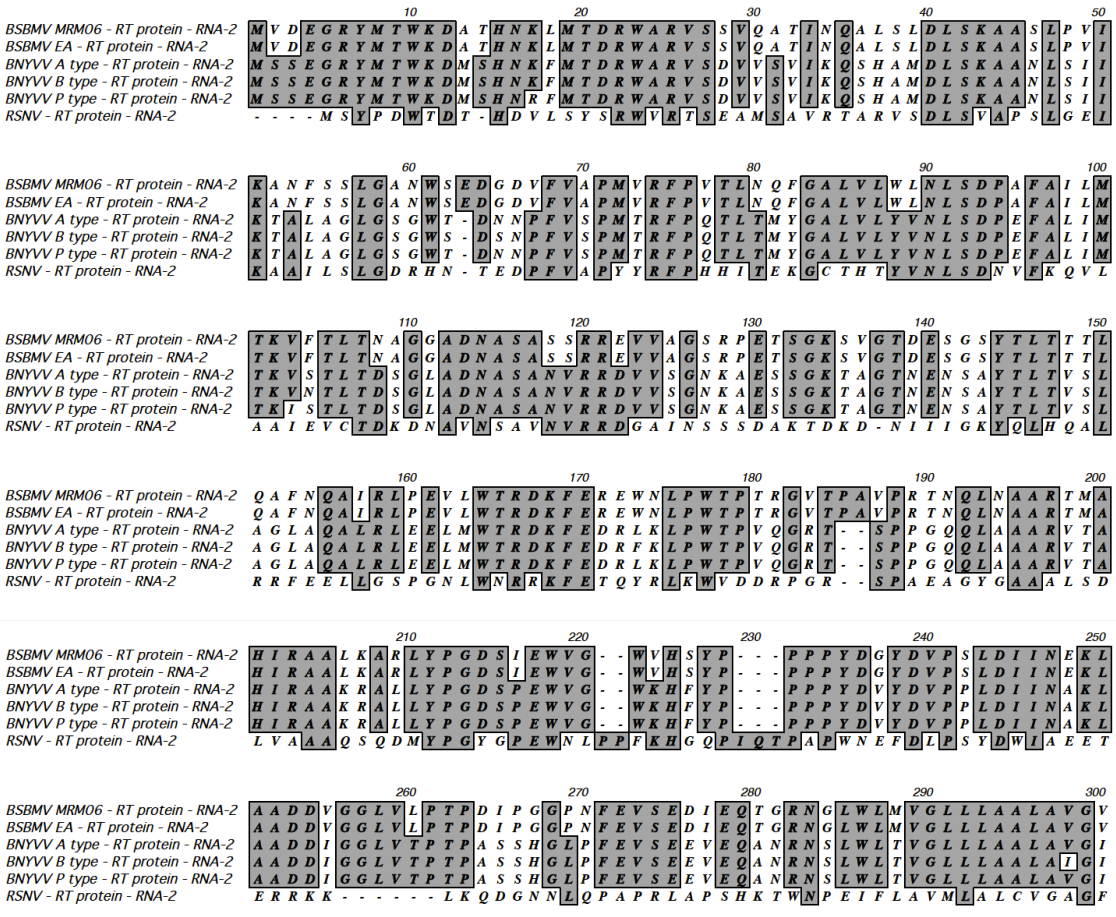
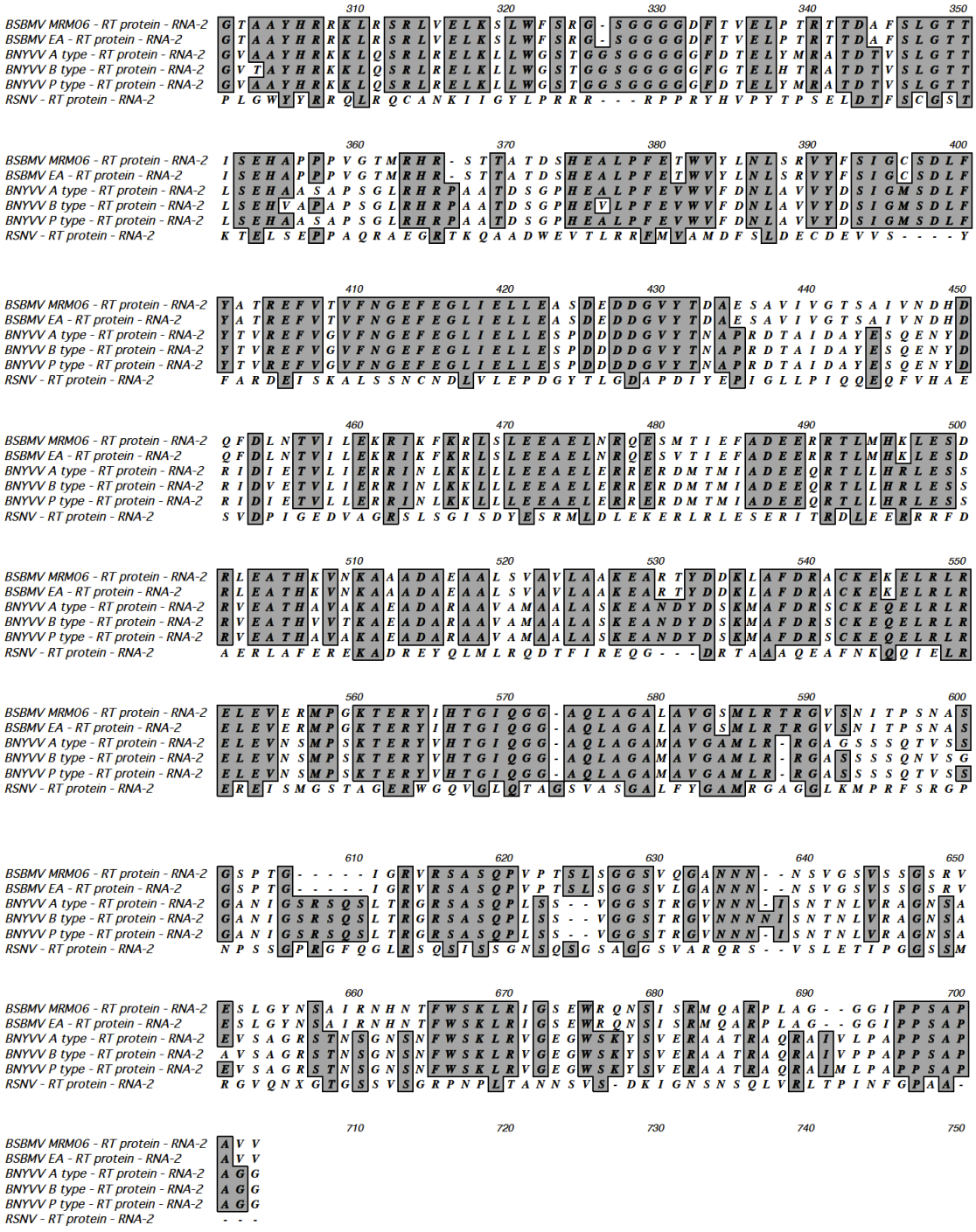
Benyvirus RNA-2 ORF1 – CP protein



Benyvirus RNA-2 ORF2 – RT protein




BSBMV MRM06 - RT protein - RNA-2
BSBMV EA - RT protein - RNA-2
BNYVV A type - RT protein - RNA-2
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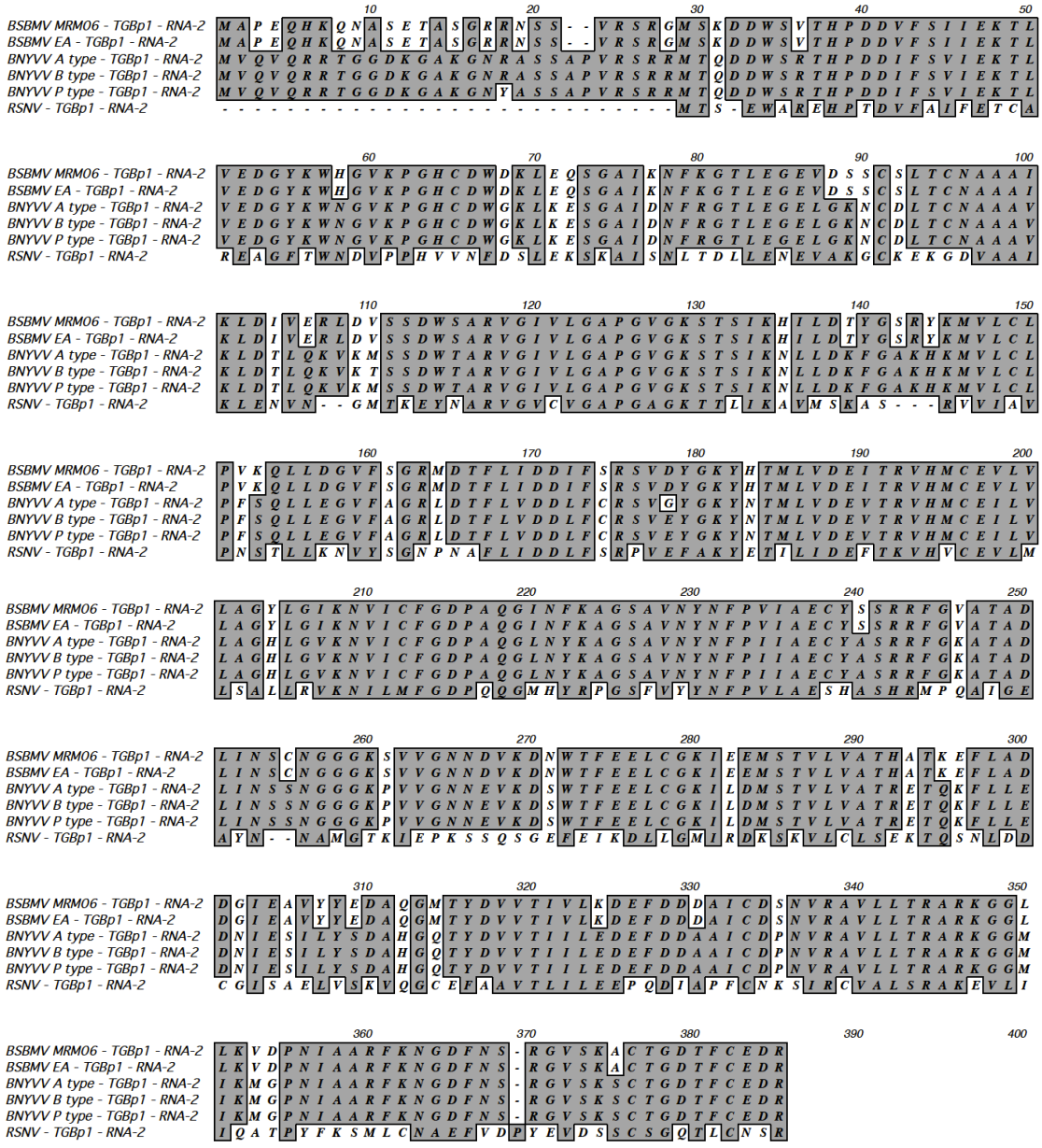
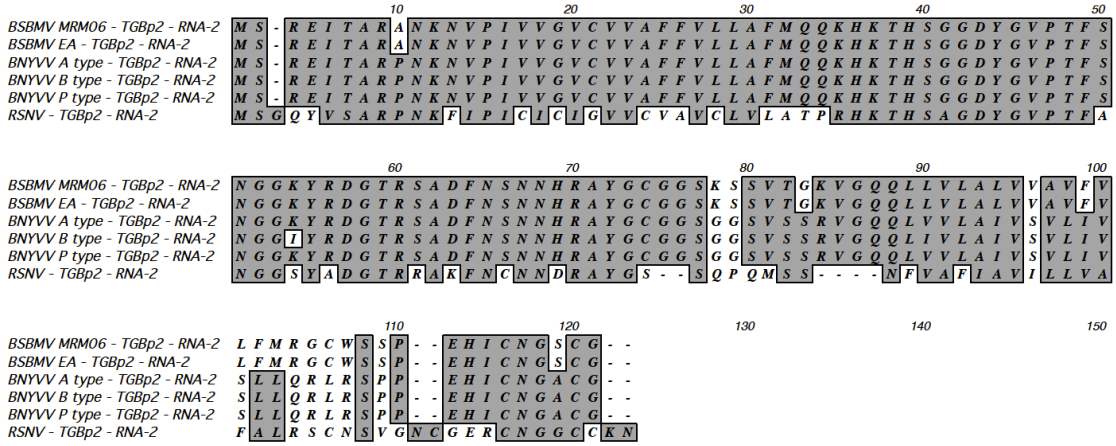
BNYVV P type - RT protein - RNA-2
RSNV - RT protein - RNA-2

Benyvirus RNA-2 ORF3 – TGBp1 proteins

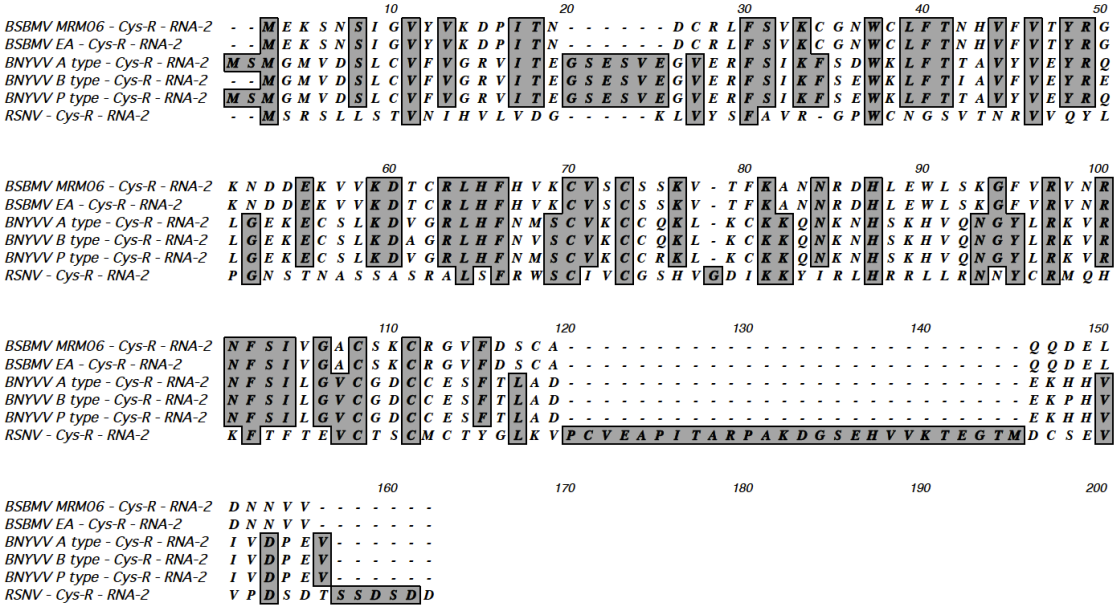


Benyvirus RNA-2 ORF4 – TGBp2 proteins

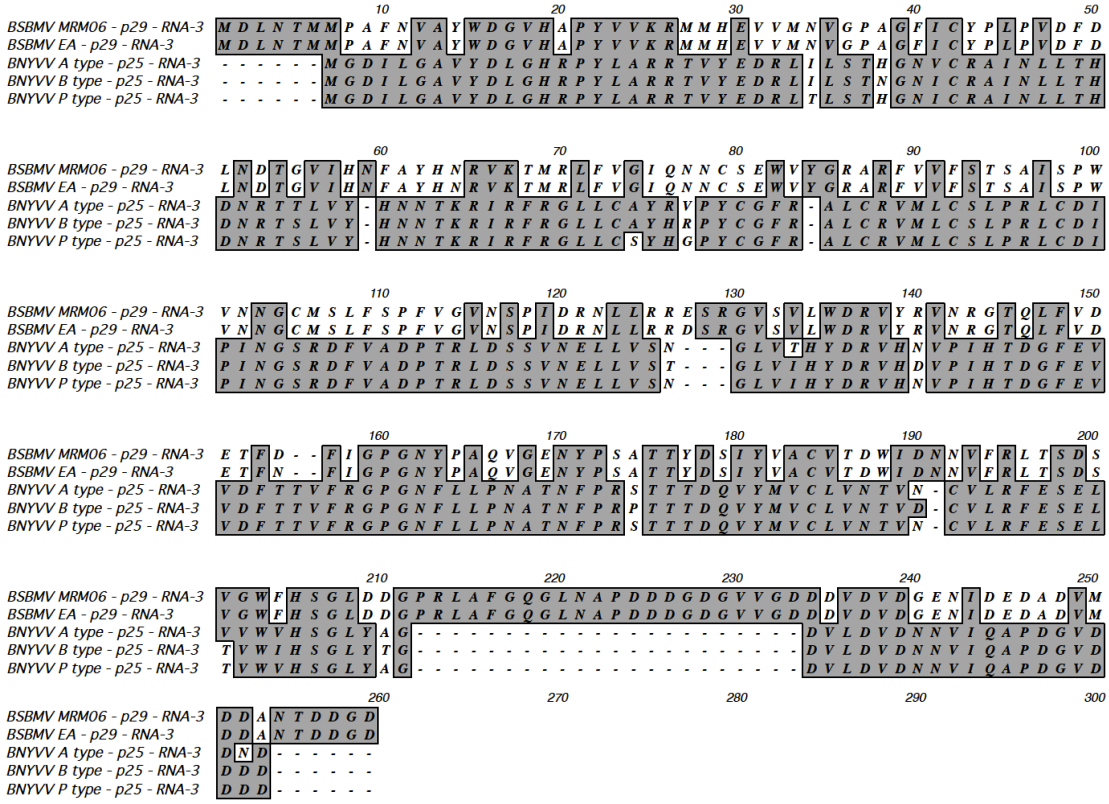


Benyvirus RNA-2 ORF5 – TGBp3 proteins

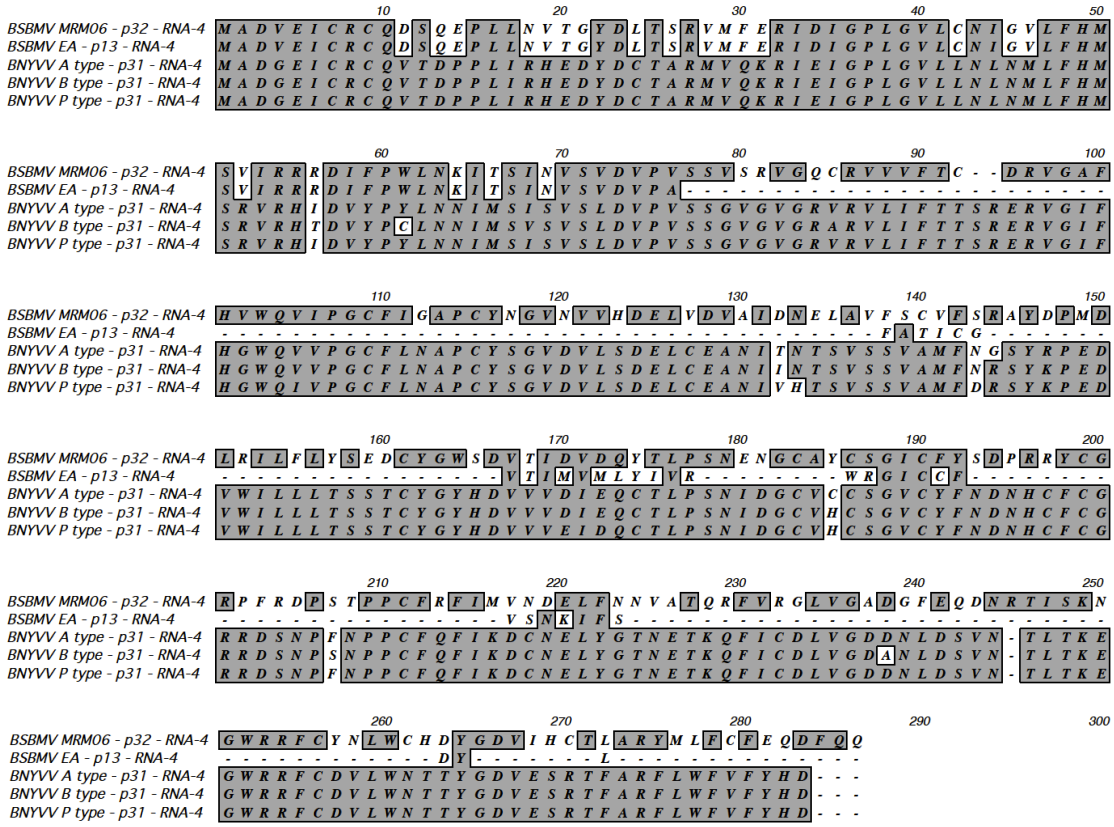
Benyvirus RNA-2 ORF6 – Cys-R proteins



RNA-3 – ORF1 proteins



RNA-4 – ORF1 proteins



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